
Prevalence of the main viruses infecting banana in La Réunion

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MASTER BIOINGÉNIEUR EN SCIENCES AGRONOMIQUES**

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CO-PROMOTEURS : PROFESSEUR SEBASTIEN MASSART, DOCTEUR PIERRE-YVES TEYCHENY.

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Encadrement du travail de fin d'études et financement de la mobilité

Ce travail de fin d'études a été réalisé à l'île de La Réunion (France), au sein de l'unité UMR PVBMT du Centre de coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) et du laboratoire de la santé du végétale (LSV) de l'agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (Anses), dont les bureaux et laboratoires sont situés au Pôle de Protection des Plantes (3P) à Saint-Pierre (Ligne Paradis).

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Abstract

[FR] À la Réunion, la production bananière, destinée au marché local et commercialisée par circuits courts, représente 17 % de la production fruitière. Elle contribue aux objectifs d'autonomie alimentaire et de santé publique du territoire, qui reposent notamment sur un accroissement de la consommation de produits frais produits localement. Si les dégâts causés par les ravageurs sur la production bananière réunionnaise sont bien caractérisés, la prévalence et l'impact des maladies virales demeurent largement méconnues. Or, les virus affectant les bananiers (*Musa* spp) sont des contraintes majeures pour la production de bananes, la production de matériel de plantation, la conservation et les échanges de ressources génétiques *Musa*. En l'absence de moyen de lutte curatif, le contrôle des maladies virales des bananiers repose principalement sur des approches prophylactiques telles que l'utilisation de matériel végétal garanti indemne de virus, le remplacement des plants infectés par des plants sains ("recourage") et la surveillance épidémiologique, qui nécessitent des méthodes de diagnostic fiables, performantes et aussi peu coûteuses que possible. Nous avons réalisé la première étude de prévalence des principaux virus de bananier à La Réunion. Pour cela, les techniques existantes de diagnostic immunologique, immuno-moléculaire ou moléculaire de ces virus ont été optimisées avec succès pour l'analyse de grandes séries d'échantillons, puis mises en œuvre sur 443 échantillons issus de bananiers en production commerciale, de la collection de ressources génétiques *Musa* du CIRAD et de bananiers sauvages collectés sur les bords de route. Nos résultats montrent une faible prévalence du BanMMV (2,71%), du BSGFV (1,58%), du BSMYV (2,26%) et du BSOLV (0,45%) dans les échantillons analysés et constituent la première observation du BanMMV à La Réunion. Ils indiquent que les bananiers sauvages sont des réservoirs potentiels de BSGFV et de BSMYV. L'analyse moléculaire des isolats de BanMMV de la Réunion a montré que le niveau de diversité moléculaire de ces isolats est significativement inférieur à celui observé en Guadeloupe ou dans le reste du monde, et qu'il n'y a pas de structuration géographique de cette diversité à la Réunion. En outre, le BBTV, le BBrMV, le BSIMV et le CMV n'ont pas été détectés dans les échantillons analysés.

[EN] On Reunion Island, banana production, intended for the local market and sold through short distribution channels, represents 17% of fruit production. It contributes to the region's objectives of food self-sufficiency and public health, which rely on increased consumption of locally-produced fresh foodstuff. The damage caused by pests to banana production in Reunion is well documented, but the prevalence and impact of viral diseases remain largely unknown. Yet viruses affecting banana plants (*Musa* spp) are major constraints for the production of fruits and planting material, and for the conservation and exchange of *Musa* germplasm. Since no curative means are available against plant viruses, the control of viral diseases of banana relies mainly on prophylactic approaches such as the use of certified virus-free planting material, the replacement of infected plants by healthy ones and epidemiological surveillance, all of which require reliable, high-performance diagnostic methods that are as inexpensive as possible.

We report on the first prevalence study of the main banana viruses in La Réunion. Existing immunological, immunomolecular and molecular diagnostic techniques for these viruses were successfully optimised for the analysis of large sample series, and implemented on 443 samples collected from banana plants in commercial production plots or in CIRAD's *Musa* genetic resources collection and from wild banana plants collected on roadsides. Our results show a low prevalence of BanMMV (2.71%), BSGFV (1.58%), BSMYV (2.26%) and BSOLV (0.45%) in the analysed samples, and constitute the first observation of BanMMV in La Réunion. They indicate that wild banana plants are potential reservoirs of BSGFV and BSMYV. Molecular analysis of BanMMV isolates from La Réunion showed that the level of molecular diversity of these isolates is significantly lower than in Guadeloupe or the rest of the world, and that there is no geographical structuring of this diversity in La Réunion. In addition, BBTV, BBrMV, BSIMV and CMV were not detected in the analysed samples.

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1. State of the art

1.1. Worldwide economic and social importance of banana

Banana is the third most cultivated tropical fruit worldwide. It is cultivated in more than 130 countries and the annual world production amounts to 124 million tons (Mt) (FAOStat, 2021). Most of this production (85%) relies on cooking types. It is consumed locally and provides staple food to hundreds of millions of people in the intertropical zone, playing a significant role in global food security. Dessert banana is the world's most exported fruit, whose production and trade generate significant income. It is a major source of employment, mainly in the rural areas of exporting countries (CIRAD, 2022). Banana can be produced all year round and, therefore, is an attractive perennial crop for farmers in developing countries as it provides a steady cash income and/or a supply of nutritious food. Banana is often grown in association with other cash crops, such as coffee (Oduol et al., 1990) or subsistence crops, such as mango, cabbage or coconut (Sarkar et al., 2022), to which banana provides protection and shade (Lassois et al., 2009).

1.2. Taxonomy, origin, geographical distribution, propagation, botanical description and ecology of banana

1.2.1. Botanical description and life cycle

Banana belongs to the genus *Musa* in the family *Musaceae* (order *Zingiberales*). Species within the genus *Musa* have no lignified parts. Hence they are not trees but giant perennial monocotyledonous herbs whose pseudostems are formed by leaf sheaths (Figure 1). New leaves arise from a meristem near ground level and push up through the pseudostem in a tight roll. Leaves have a large petiole and lamina with a stout midrib and numerous parallel veins extending to the margin. Their surface can reach several square metres.

Flowering is initiated when the apical meristem stops producing leaves and forms an inflorescence, generally after 25-50 leaves have been produced and 10-15 functional leaves are in place to ensure an active photosynthetic activity (Jones, 2000). Bananas produce a single bunch over its lifetime. Therefore, once the bunch is harvested, cultivated bananas are chopped and one of the suckers produced from the crown by vegetative multiplication is selected to take over and grow a new plant that will produce another bunch. Selecting a sucker to produce a daughter plant reduces competition for water and nutrients and fastens the next bunch's production. Moreover, young suckers that are removed during the desuckering process can be used by farmers as planting material. However, using suckers as planting material can promote the spread of pests and pathogens when suckers arise from infested/infected mother plants. This practice has been shown to cause outbreaks of several banana diseases and pests around the world (Jones, 2002).

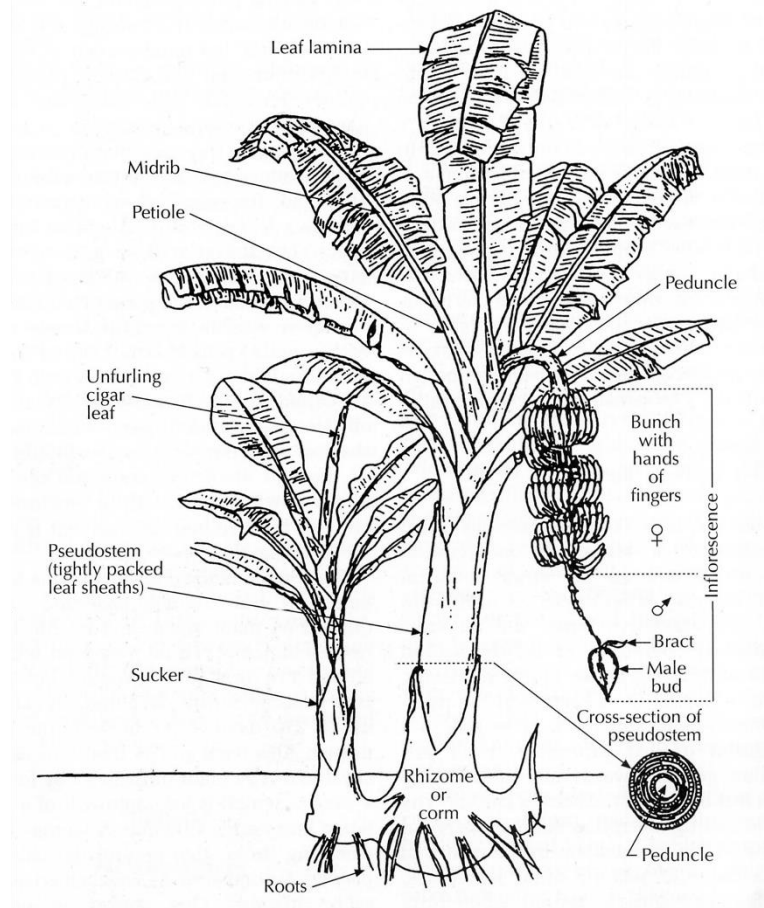


Figure 1: Representation of a banana plant.

From Champion (1963)

1.3. Origin, spread, diversity and geographical distribution of edible banana cultivars

Even if there is no straightforward botanical distinction, bananas are usually divided into two main categories: dessert bananas, which are eaten raw, and cooking bananas, which include but are not limited to plantain. Edible banana cultivars originate from wild *M. acuminata* diploid (AA) subspecies that arose in South-East Asia (Simmonds, 1962) and underwent hybridisation in South East Asia and western Melanesia after they were brought into contact by human movements (Perrier et al., 2011). Interspecific hybridisation between diploid *M. acuminata* landraces and diploid *M. balbisiana* (BB) also occurred in South East Asia, leading to interspecific hybrids. Over a thousand banana cultivars have been listed, showing a remarkable diversity (Figure 2) in fruit size, colours, tastes and starch content. However, the genetic diversity of *Musa* spp is relatively narrow.

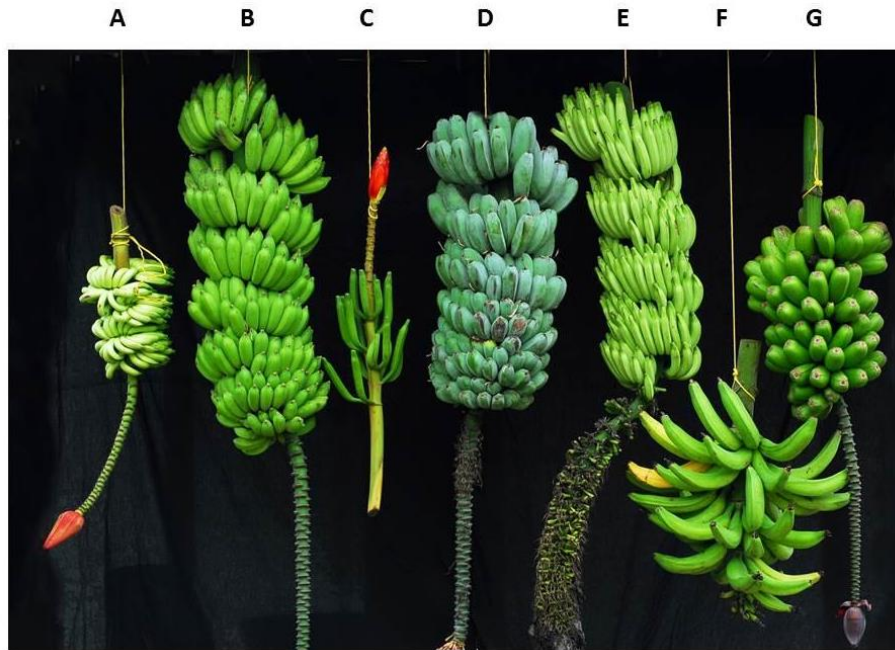


Figure 2: Morphological diversity of banana fruits.

A: Selangor (*Musa acuminata* subsp. Malaccensis); B: Pisang Ceylan (AAB); C: *Musa becarii* (ornamental); C: Butuhan (wild BB); D: Pisang Jari Buaya (AA cv); E: Curare Enano (AAB plantain type); F : Popoulou (AAB).

© P. Fournier, CIRAD

Human migrations and trade shaped the worldwide dissemination of banana cultivars (Lassoudière, 2007), especially in Africa and Latin America (Kumar et al., 2015). Many domesticated cultivars are natural hybrids with triploid genomes, such as dessert bananas of the Cavendish type (AAA) and plantain (AAB or ABB). Edible cultivars were selected for parthenocarpy, resulting in varieties that produce seedless fruits. More recently, breeding programmes were established to create new varieties with disease resistance or drought tolerance traits. However, low fertility or sterility of many cultivars makes the breeding of *Musa* spp. challenging. (Ortiz, 2013)

There is a wide range of cultivars of cooking bananas grown for local consumption, and they differ according to geographical areas. For example, the East African highland banana (AAA) is specifically grown in the African Great Lakes region of Uganda, Tanzania, Kenya, Rwanda and Burundi (Ochola et al., 2022). Conversely, a single cultivar of dessert banana, Grande Naine from Cavendish (AAA) cultivar's group, is grown worldwide for export. The whole supply chain has been standardised for this cultivar, making the introduction of new varieties on the export market difficult.

2. Main diseases and pests of banana

Diseases and pests are significant issues for crops, including bananas, because they impact yields and/or quality. Over the last century, several banana diseases have emerged and spread from their area of origin to major production zones due to human migration and the exchange of infected banana plants (Drenth et al., 2021), prompting efforts to develop and implement control strategies. This is especially true for dessert banana, which relies on a single group of cultivars, Cavendish, that are highly sensitive to several devastating diseases such as banana bunchy top disease (BBTD) or Fusarium wilt. Table 1 summarises the main diseases and pests of bananas.

Table 1: Origin and current distribution of the most important banana diseases and pests

Disease/pest	Pathogen	Area of origin	Current distribution
Fusarium wilt race 1	<i>Fusarium oxysporum</i> sp. <i>cubense</i> race 1	Southeast Asia	Pantropical
Fusarium wilt tropical race 4	<i>F. oxysporum</i> sp. <i>cubense</i> tropical race 4 (<i>F. odoratissimum</i>)	Southeast Asia	Asia, Australia, Africa, Middle East, Europe, Colombia
Yellow Sigatoka	<i>Pseudocercospora musae</i>	Southeast Asia	Pantropical
Black leaf streak	<i>Pseudocercospora fijiensis</i>	Southeast Asia	Pantropical, (excluding Australia)
Freckle	<i>Phyllosticta cavendishii</i>	Southeast Asia	Southeast Asia (excluding Australia)
Banana bunchy top	<i>Banana bunchy top virus</i>	Southeast Asia	Asia, Australia, Africa
Blood disease	<i>Ralstonia syzygii</i> subsp. <i>celebesensis</i>	South Sulawesi	Indonesia, Peninsular Malaysia
Moko	<i>Ralstonia solanacearum</i>	Costa Rica, Amazonia	Central and South America, the Philippines, Malaysia
Xanthomonas wilt	<i>Xanthomonas vasicola</i> pv. <i>musacearum</i>	Ethiopia	Central Africa
Weevil borer	<i>Cosmopolites sordidus</i>	Malaysia, Indonesia	Central Africa, Central America, Brazil, the West Indies, Eastern Australia and on many Indian and Pacific Ocean islands
Nematodes	<i>Radopholus similis</i> , <i>Pratylenchus coffeae</i> , <i>Helicotylenchus multicinctus</i> , and <i>Meloidogyne</i> spp.		Pantropical

Diseases and pests cause direct losses due to reduced production and increased costs associated with the control of these diseases and pests, such as the production and control of virus-free planting material (Kumar et al., 2015).

2.1. Pests

2.2. Banana weevil

Cosmopolites sordidus (Germar) (Figure 3-A) is a phytophagous Coleoptera from the *Curculionidae* family specific to *Musa sp.* It is present worldwide in all banana-growing areas (CABI, 2021). To lay eggs, adult females bore holes into banana corms. After a week, larvae hatch and burrow tunnels to feed (Figure 3-B), weakening the root system and increasing the risk that banana plants are being blown over under windy conditions. Infestations reduce plant growth and cause yellowing of the leaves. *C. sordidus* rarely fly and are mainly spread over long distance through the use of infested plant material (Treverrow, 2003). Pesticides are efficient against *C. sordidus*, but they cause environmental concerns. Alternative methods include pheromone traps, which are effective in the field when infestation rates are low to moderate, and biological control methods using entomopathogenic fungi, nematodes or predators, which have been shown to be effective in the laboratory and under controlled conditions, but are not yet being used in practice (Tresson et al., 2021).

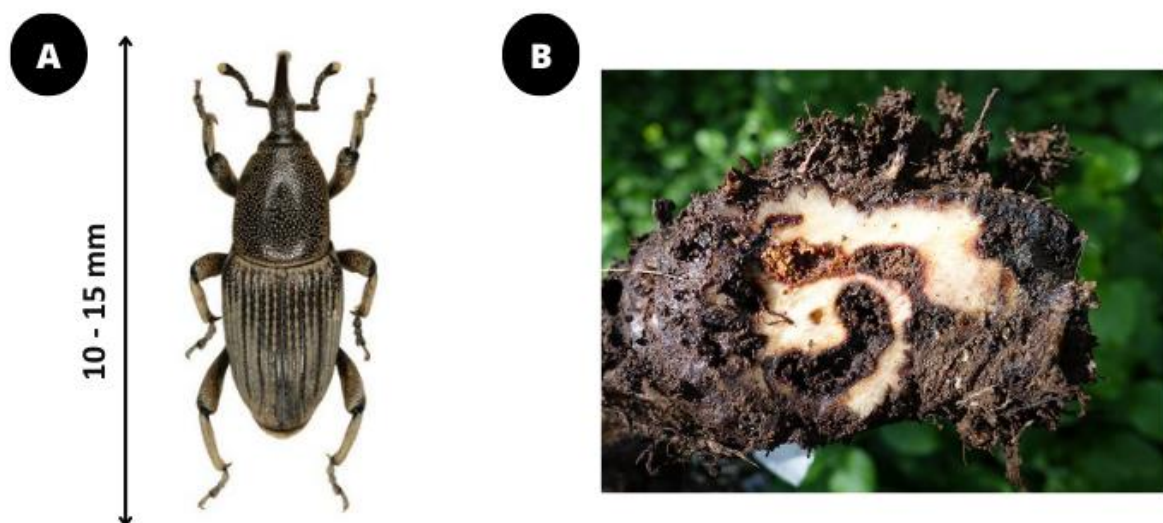


Figure 3: Adult specimen and damage caused by *Cosmopolites sordidus* (Germar) on *Musa Spp.*

A: Adult specimen © J.Rheinheimer; B: Galleries dug by *C.sordidus* larvae in a banana bulb © Scot Nelson.

2.3. Nematodes

Several nematode species are endoparasites of banana roots and corm, of which *Radopholus similis* (Cobb, 1893) (Figure 4-A), *Pratylenchus coffeae* (Zimmerman, 1898), *Helicotylenchus multicinctus* (Cobb, 1893), and *Meloidogyne* spp. are the most damaging (Gowen et al., 1990). They are present worldwide in all banana-growing areas. Nematodes destroy parenchyma

cells and cell walls of the root system during feeding, creating tunnels that become necrotic and induce the destruction of roots (Figure 4-B). Infestations lead to decreases in water uptake and mineral nutrition, reducing plant growth and fruit yields (Gowen et al., 1990). They also weaken the root system and, like *C. sordidus*, increase the risk of being blown over under windy conditions. Efficient control methods used to involve nematicides, of which most are now banned in many countries due to high toxicity. Alternative control measures include cultural practices such as fallows, whose purpose is to reduce infestation levels in the soil, and the use of nematode-free planting material such as in vitro plants.

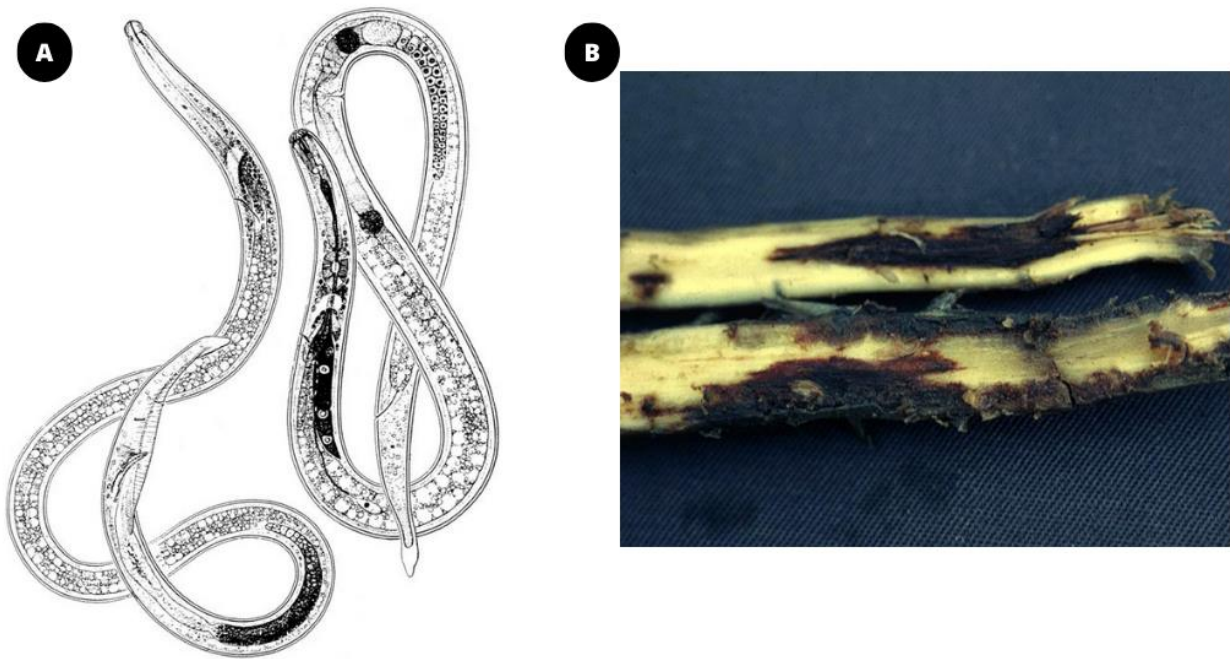


Figure 4: Adult specimens and symptoms of *Radopholus similis* (Cobb, 1893) on *Musa Spp.*
A: From Siddiqi, 1985, *R. similis*. male and female.; B: Root rot symptoms in banana roots. ©Michael McClure, University of Arizona, Bugwood.org

2.4. Fungal diseases

2.4.1. *Fusarium oxysporum sp. cubense* (FOC)

Fusarium wilt (Panama disease) is caused by the soil ascomycete fungus *Fusarium oxysporum sp. cubense* (FOC), of the family *Nectriaceae*. It was first isolated and described by E.F Smith in 1910 (Smith, 1910) and since then, Foc has been divided into pathotypes called “races” (Garcia-Bastidas, 2022). Each race has been identified under certain conditions and can cause a wide range of vascular damage to banana (Ploetz, 2015). For example, tropical race 1 (TR1) caused a major outbreak on highly sensitive cultivar Gros Michel, which dominated the international trade of desert banana but was replaced worldwide by cultivars of the Cavendish group, which are resistant to TR1.

F. oxysporum invades the vascular system through the roots, then spreads to the rhizome and the pseudostem (Ploetz, 2015), causing leaf yellowing (Figure 5-A), wilt and vascular necrosis

in the pseudostem (Figure 5-B) (Garcia-Bastidas, 2022). The fungus produces conidia and chlamydozoospores that can be spread via surface (including irrigation) and runoff water and survive in soils for decades, providing fast, efficient and long-lasting transmission, and making it almost impossible to eradicate. Long distance spread of the fungus is through the introduction of infected germplasm. Tropical race 4 (TR4) is the most recently described race of *F. oxysporum*. It has a broader host range than previously described races and the ability to infect cultivars that are resistant to other races, including those of the Cavendish group. TR4 was confined to South East Asia and northern Australia until the 1990's and has since spread rapidly across Asia, to the Middle East, the Indian subcontinent, Africa and recently to South America. It is currently the biggest threat to banana production worldwide, prompting efforts to control its spread and breed new varieties for resistance (Chen et al., 2019). Current prophylaxis methods are limited to and rely on the use of clean planting material and strong quarantine regulations.



Figure 5: Symptoms of Fusarium wilt.

A: General necrosis caused by *F. oxysporum* TR4 © Gustavo Martinez; B: Internal brown to red brick discoloration of the vascular system © Gustavo Martinez.

2.5. Sigatoka leaf streak diseases

Yellow and Black Sigatoka leaf spot diseases (SD and BLSD, respectively) and Eumusae leaf spot are foliar diseases caused by ascomycete fungi species *Pseudocercospora musae* (Zimm.) Deighton, 1976, *Pseudocercospora fijiensis* (M. Morelet) Deighton, 1976 and *Pseudocercospora musae* (Zimm.) Deighton, 1976, respectively (Drenth et al., 2021). All three fungi produce ascospores and conidia, ensuring an efficient sexual and asexual reproduction.

Conidia are spread by water and wind. High temperatures (25-28°C) and high relative humidity favour the germination of spores and hasten symptom appearance (Jacome et al., 1992).

SD causes lesions on leaves that become necrotic, resulting in lower yields. It is not a major concern for growers. On the opposite, BSD and Eumusae leaf spot disease cause more severe symptoms and infect more banana varieties (Guzman et al., 2018). Early symptoms of BSD and Eumusae leaf spot disease are very similar, causing tiny, chlorotic spots on the leaves (Figure 6). The spots grow into thin brown streaks and large areas of the leaves become necrotic and water-soaked (Bennett et al., 2003), resulting in a significant decrease in photosynthesis and fruit yield. Cultivar Cavendish is particularly sensitive to BSD and Eumusae leaf spot disease, which are the major constraint for the production and export of dessert banana. Systemic fungicides have been used extensively for controlling BLSD and Eumusae leaf spot, although they require frequent (up to 50) applications (Castillo-Arévalo, 2022), they are detrimental to human and environment health and promote the selection of resistant fungi populations (Oliveira et al., 2022). They are still being used in Africa and South America, but have been banned from EU countries and territories such as the French overseas departments of Guadeloupe, Martinique, La Réunion and Mayotte where the control of SD and BLSD now relies on manual removal of infected leaves upon symptom appearance. Breeding resistant cultivars against SD and BLSD is a more sustainable approach that has been successfully implemented by several breeding programmes worldwide, including those of the Honduran Foundation for Agricultural Research (FHIA) and CIRAD. For example, CIRAD developed *Pointe d'Or*[®] (CIRAD 925), a resistant variety that has been distributed in the French West Indies where its fruits are commercialised (De Bellaire et al., 2010; CIRAD, 2019).

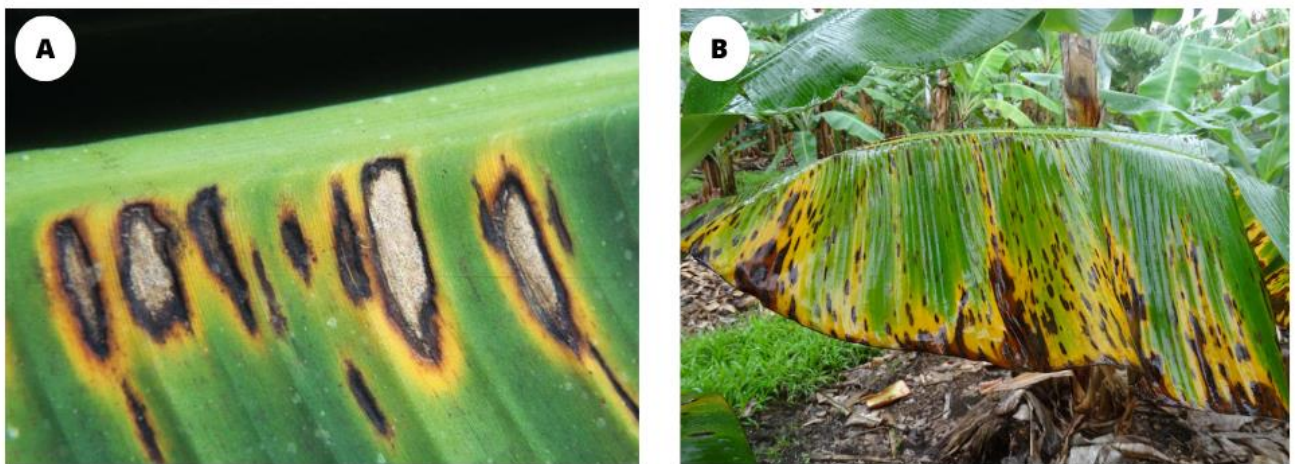


Figure 6: Black sigatoka symptoms on *Musa Spp.*

A : Chlorotic spot that become necrotic © Scot Nelson ; B: Necrotic spots turning into brown streak © Scot Nelson.

2.6. Bacterial diseases

2.6.1. Moko disease

Moko disease is a devastating disease caused by an ecotype of *Ralstonia solanacearum* (Peeters et al., 2013), a soil-borne bacterium with a high genetic diversity and a broad host range, including tomato, potato and banana (Albuquerque et al., 2014). Typical Moko disease symptoms vary depending on strains and include yellowing and wilting of the leaves (Figure 7-A), internal rot and vascular discolouration in the pseudostem (Figure 7-B) (Drenth et al., 2021). Infected plants produce deformed fruits that turn black and rot (Figure 7-C).

R. solanacearum is spread from infected plant parts (roots, stems, bunches, fruit, peel, suckers or leaf material). It persists in the soil for extended periods (over 18 months), allowing the spread of the disease through infected soil.

Control of *R. solanacearum* is difficult. Antibiotics are forbidden practically far and wide as they are not very efficient and their use in the field is prohibited because it would promote antibiotic resistance. The use of bacteriophages could be a promising alternative (Ramírez et al., 2020). Current control measures include the use of pathogen-free planting material, regular decontamination of tools and general prophylaxis.

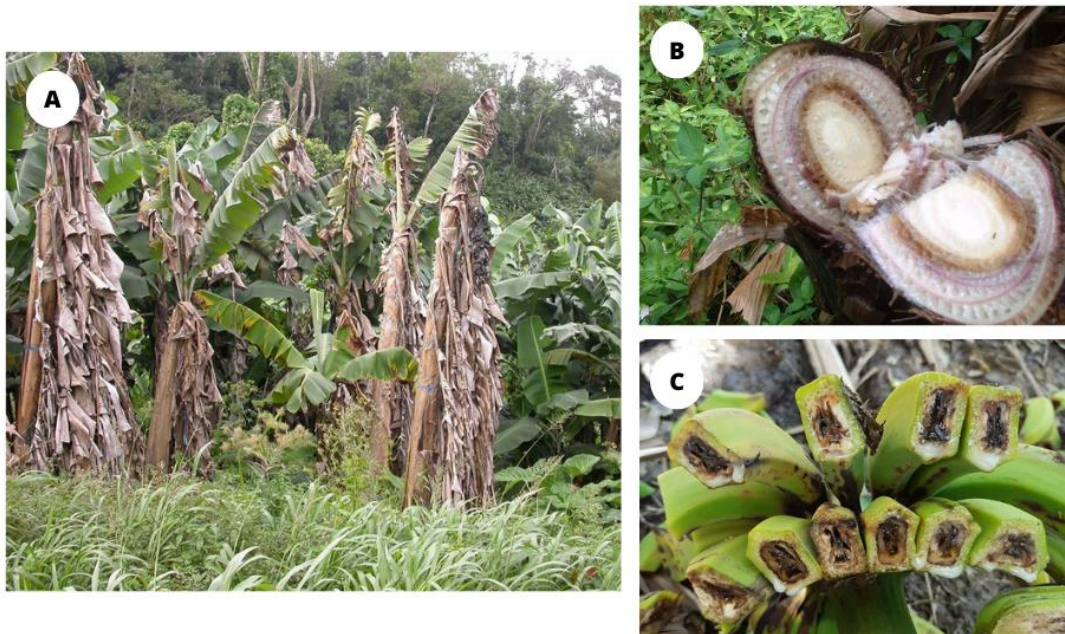


Figure 7: Symptoms of Moko disease caused by *R. solanacearum* on *Musa spp.*

A: collapsing Cavendish bananas ©Emmanuel Wicker ; B: internal rot and vascular discolouration in the pseudostem © Adriano Freire Silva ; C: Brown rots inside unripe banana fruits © Adriano Freire Silva

2.6.2. Banana xanthomonas wilt (BXW) disease

Banana xanthomonas wilt (BXW) disease is caused by *Xanthomonas campestris* pv. *musacearum* (Xcm). Symptoms of BXW can be confused with those of Fusarium wilt. They include internal discolouration and premature ripening of the fruit (Figure 8-A-C), yellow-

orange discolouration of the vascular bundles (Figure 8-B) and dark brown tissue scarring, wilting and yellowing of the leaves, wilting of the bracts and shrivelling of the male buds (Figure 8-A). All leaves may wilt, causing the death of infected plants. More specific symptoms include the excretion of bacterial ooze from plant organs. BXW is spread by insects that visit the male inflorescences, contaminated tools and planting material. Control strategies include the removal of male flowers after pollination has occurred, selection of resistant cultivars or varieties without "male bells" and with dehiscent bracts (Drenth et al., 2021). BXW is a very destructive disease with an important impact in Uganda and the great lake region of East Africa (Ocimati et al., 2019).

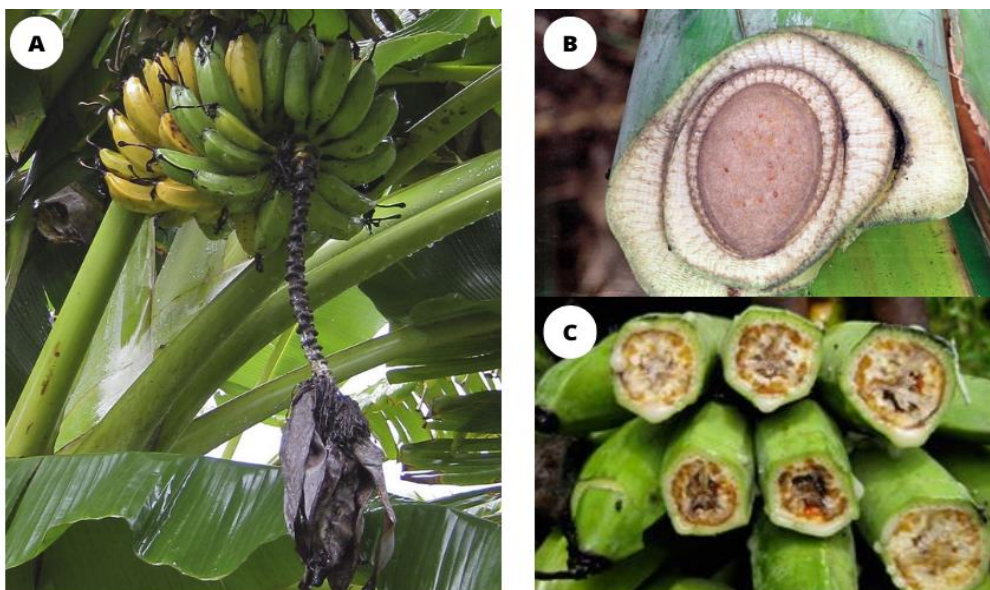


Figure 8: Symptoms of banana xanthomonas wilt (BXW) disease on *Musa spp.*

A: Shrivelled male bud and uneven ripening ©Guy Blomme; B: Internal discolouration in the central core of the stem © Pascal Lepoint ; C: Rusty-brown straining © IITA (International Institute of Tropical Agriculture)

2.7. Viral diseases

About 20 viruses belonging to five families have been reported on bananas worldwide. Table 2 provides an overview of the taxonomy, transmission and diagnosis of the main viruses infecting bananas.

Overall, viruses are not a major constraint for banana production worldwide, except banana bunchy top virus (BBTV), which is one of the most destructive diseases affecting banana. BBTV is widespread in several regions and countries in Asia, Africa and the Pacific where it causes important yield losses and threatens food security. Viruses are readily transmitted by vegetative propagation. Vegetatively propagated crops such as bananas accumulate viruses over time because they lack sexual reproduction, which acts as natural sanitation because most plant viruses are not seed-transmitted. Therefore, viruses are a major constraint for the exchange of *Musa* germplasm at local, regional, and international scales prompting efforts to mitigate the risks of spreading diseases through infected germplasm (Thomas, 2015). One of

the most successful control measures relies on the use of virus-free planting material such as vitroplants.

Table 2: Characteristics of the main viruses reported on banana

Virus Name	Taxonomy			Transmission		References
	Acronym	Family	Genus	Vector-borne transmission	Other	
Abacá bunchy top virus	AbBTV	<i>Nanoviridae</i>	<i>Babuvirus</i>	Non-persistent, by aphid species <i>P. nigronevosa</i>	Infected plant material, vegetative propagation	Sharman <i>et al.</i> , 2008
Banana mild mosaic virus	BanMMV	<i>Betaflexiviridae</i>	<i>Banmivirus</i>	Unknown	Infected plant material, vegetative propagation	Gambley & Thomas, 2001; Teycheney <i>et al.</i> , 2005a
Banana bract mosaic virus	BBrMV	<i>Potyviridae</i>	<i>Potyvirus</i>	Non-persistent, by several aphid species	Infected plant material, vegetative propagation	Thomas <i>et al.</i> , 1997
Banana bunchy top virus	BBTV	<i>Nanoviridae</i>	<i>Babuvirus</i>	Circulative non-propagative, by aphid species <i>P. nigronevosa</i>	Infected plant material, vegetative propagation	Dale, 1987
Banana streak viruses*	BSV	<i>Caulimoviridae</i>	<i>Badnavirus</i>	Semi persistent, by several mealybug species	Infected plant material, vegetative propagation, infectious eBSVs	Martinez Mota, 2015
Banana virus X	BVX	<i>Betaflexiviridae</i>	Unassigned	Unknown	Infected plant material, vegetative propagation	Teycheney <i>et al.</i> , 2005
Cucumber mosaic virus	CMV	<i>Bromoviridae</i>	<i>Cucumovirus</i>	Non-persistent, by several aphid species	Infected plant material, vegetative propagation	Jones <i>et al.</i> , 2000; Jones, 2002
Sugarcane mosaic virus, abacá strain	SCMV-Ab	<i>Potyviridae</i>	<i>Potyvirus</i>	Non-persistent, by several aphid species	Infected plant material, vegetative propagation	Gambley <i>et al.</i> , 2004

*Complex of 9 different species recognized by ICTV; PDO-RT-PCR = Polyvalent Degenerate Oligonucleotide RT-PCR

Our study focuses on banana bunchy top virus (BBTV), cucumber mosaic virus (CMV), banana bract mosaic virus (BBrMV), banana mild mosaic virus (BanMMV), and banana streak viruses (BSVs: BSOLV, BSGFV, BSIMV, BSMYV), whose main characteristics are summarised below.

2.7.1. *Banana bunchy top virus*

Banana bunchy top virus (BBTV) is a ssDNA virus belonging to the genus *Babuvirus* in the family *Nanoviridae* (Thomas et al., 2021). It causes banana bunchy top disease (BBTD), the most devastating viral disease of bananas, causing up to 100% losses. In some areas of Africa, banana is no longer grown because of the prevalence of BBTV. BBTV is a regulated quarantine disease in most banana growing areas, leading to a very tight surveillance.

BBTV was first reported on Cavendish banana in Fiji at the end of the 19th century (Magee, 1927). Since then, the virus has spread to Asia, Australia, Africa and the Pacific, most likely through the introduction of infected planting material.

BBTV causes stunting (Figure 9-A), characteristic “morse-code” pattern on leaves (Figure 9-B-C) and, ultimately, the death of infected plants. Plants infected at an early stage generally do not produce a bunch (Thomas et al., 2003) and plant death occurs in a few months. If the infection occurs later during the growing cycle, infected plants may bear fruit once before dying, although fruits will be small and distorted.

BBTV is transmitted by the aphid species *Pentalonia nigronervosa* Coquerel on a circulative non-propagative mode. Once acquired through feeding on an infected plant, BBTV is retained by the vector during its entire lifespan, after a minimum acquisition-access period of 4 hours and a minimum inoculation-access period of 15 minutes (Hu et al., 1996). The exchange of infected planting material also spreads BBTV.

There is no curative treatment to control BBTV once it has infected a banana plant. Implementing strict domestic regulations, epidemiological surveillance, promoting the use of virus-free planting material such as vitroplants and systematic eradication of infected plants can help prevent the spread of the disease. Natural sources of resistance were identified recently, offering prospects for breeding improved resistant varieties (Cueva et al., 2022).

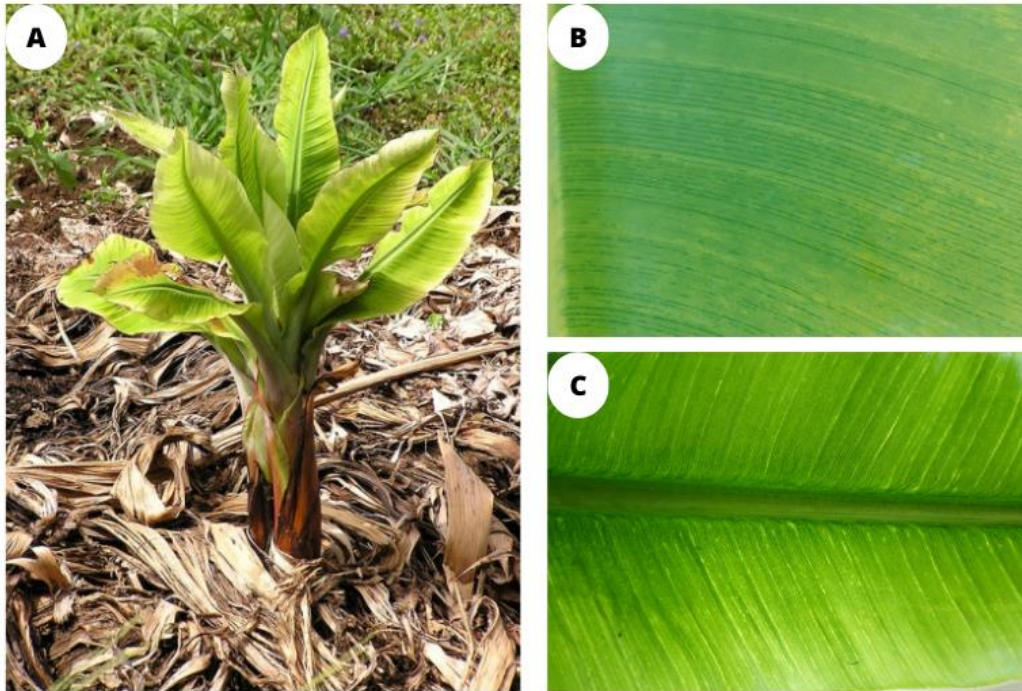


Figure 9: Symptoms of banana bunchy top virus (BBTV).

A: Symptoms of banana bunchy top virus on Cavendish banana ©Scot Nelson. ; B - C: BBTV characteristic “morse-code” pattern on banana leaves ©University of Hawai, CTAHR © Scot Nelson.

2.7.2. *Banana bract mosaic virus*

Banana bract mosaic virus (BBrMV) belongs to the genus *Potyvirus* within the family *Potyviridae* (Inoue-Nagata et al., 2022). It has been reported in India (Selvarajan et al., 2016), the Philippines, Ecuador (Quito-Avila et al., 2013), Western Samoa, Thailand and Vietnam (Balasubramanian et al., 2014).

The most characteristic symptom of early infection by BBrMV are greenish yellow streaks which later turn into dark brownish-red necrosis on the stem (Figure 10-A), the leaf lamina and veins (Figure 10-B). Symptoms are visible on newly emerged leaves when the older leaf sheaths are pulled away from the pseudostem. More advanced and typical symptoms are flower bracts mosaics (Figure 10-B).

BBrMV is transmitted on a non-persistent mode by several aphid species, including *Ropalosiphum madiis* (Fitch) and *Myzus persicae* (Sulzer), and through all forms of vegetative propagules, including micropropagated plantlets, suckers and corms.

Besides *Musa* spp, BBrMV has been reported on cardamon, *Elettaria cardamomum*, (Siljo et al., 2012) and ginger, *Zingiber officinale* (Wang et al., 2010). No source of resistance is known, precluding efforts to breed resistant hybrid varieties. As for BBTV, control measures rely on the use of virus-free planting material and roguing.

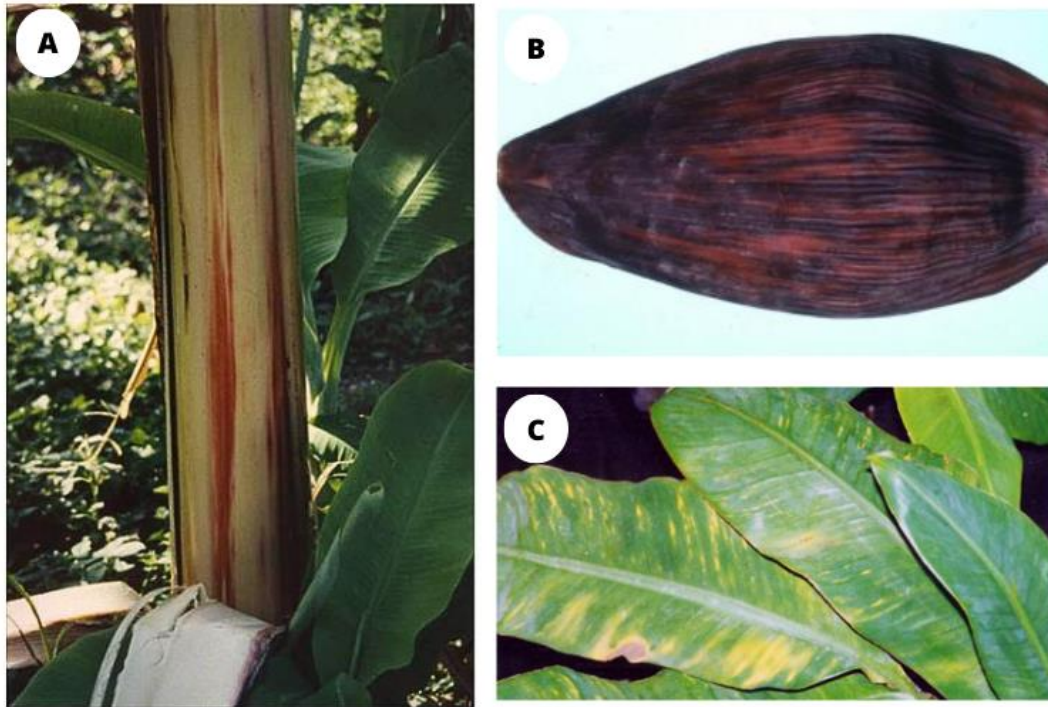


Figure 10: Symptoms of *banana bract mosaic virus* (BBrMV).

A: Red-brown, spindle-shaped streaks on the exposed banana stem ©John Thomas. ; B : A dark, red-brown mosaic pattern on the banana flower bract ©John Thomas. ; C: leaf discoloration turning into red necrosis.

2.7.3. *Banana streak viruses*

Banana streak viruses (BSVs) are members of the genus *Badnavirus* in the family *Caulimoviridae* (Teycheney et al., 2020). Nine species of BSVs are currently recognized by the International Committee for the Taxonomy of Viruses (ICTV; Table 3 and <https://ictv.global/report/chapter/caulimoviridae/caulimoviridae/badnavirus>). BSVs are reported to be the most widely distributed viruses infecting banana and plantain around the world (Kumar et al., 2015). Their impact on yield and fruit quality has not been studied extensively but is supposed to be moderate (Daniells et al., 2001; Umber et al., 2022).

BSVs cause typical chlorotic streak symptoms on the leaves (Figure 11-A) that can become necrotic upon co-infections by cucumber mosaic virus (CMV) or banana mild mosaic virus (BanMMV). More severe symptoms such as pseudostem splitting (Figure 11-D), necrosis of the leaf cigar, internal and external fruit necrosis can be observed on sensitive cultivars.

BSVs are horizontally transmitted on the semi-persistent mode by several mealybug species including *Planococcus citri*, *P. ficus*, *P. minor*, *Dysmicoccus brevipes*, *Paracoccus burnerae* and *Saccharicoccus sacchari*. BSVs are also spread by the use of infected planting material. Vertical transmission occurs in interspecific varieties through the activation of infectious endogenous banana streak viruses (eBSVs), which are widespread in *Musa balbisiana* progenitors (Chabannes et al., 2013). Activation is triggered by biotic and abiotic stresses, leading to spontaneous infections (Chabannes et al., 2013) Control of horizontal transmission can be achieved by the use of virus-free planting material such as vitroplants, and by replacing

infected plants by healthy ones. Improved *M. balbisiana* progenitors devoid of infectious eBSVs were obtained (Umber et al., 2016), making it possible to breed new interspecific hybrids with no risk of activating infectious eBSVs.

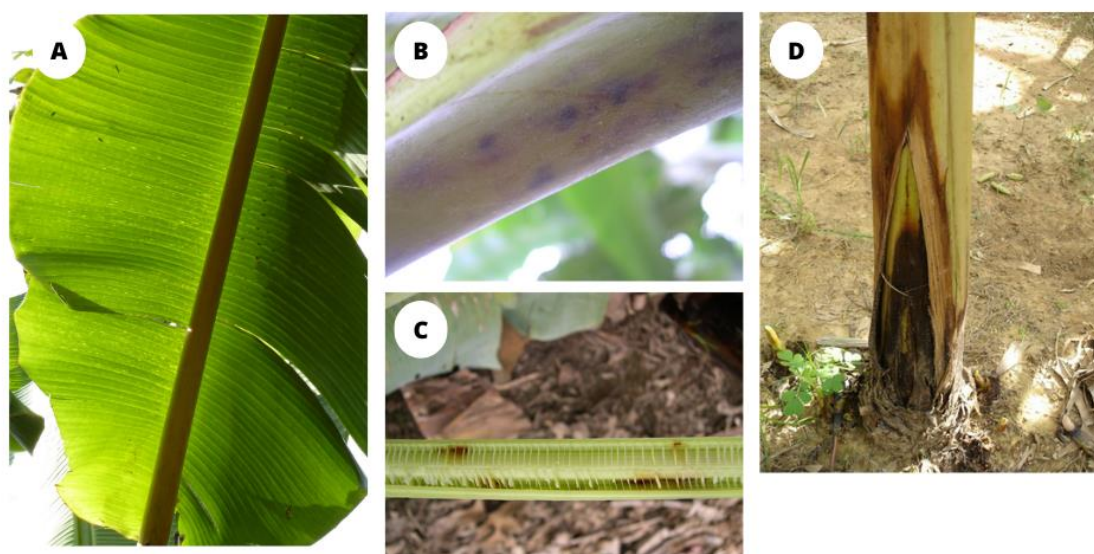


Figure 11: Typical chlorotic streak symptoms on the leaves cause by BSV infection.

A: Typical leaf chlorotic streak symptoms © P.-Y. Teycheney, CIRAD.; B - C :Dark spots on the petiole © P.-Y. Teycheney, CIRAD ; D: Pseudostem splitting cause by BSV infection © P.-Y. Teycheney, CIRAD.

Table 3: Banana streak viruses recognized by the International Committee for the Taxonomy of Viruses.

Acronym	Name	Reference
BSVNV	Banana streak Vietnam virus	Lheureux <i>et al.</i> , 2007
BSGFV	<i>Banana streak Goldfinger virus</i>	Li et al., 2020
BSIMV	<i>Banana streak Imové virus</i>	Geering <i>et al.</i> , 2014
BSMYV	<i>Banana streak Mysore virus</i>	Geering <i>et al.</i> , 2005
BSOLV	<i>Banana streak Obino l'Ewaiï virus</i>	Harper & Hull, 1998
BSUAV	<i>Banana streak UA virus</i>	James <i>et al.</i> , 2011
BSUIV	<i>Banana streak UI virus</i>	
BSULV	<i>Banana streak UL virus</i>	
BSUMV	<i>Banana streak UM virus</i>	

Reproduced from Martinez Mota (2015).

2.7.4. *Banana mild mosaic virus*

Banana mild mosaic virus (BanMMV) belongs to genus *Banmivirus* (family *Betaflexiviridae*) (Gambley et al., 2001; Silva et al., 2022). BanMMV infections are generally symptomless. However, in susceptible cultivars, leaves of infected plants may display a mild chlorotic mosaic (Jones, 2000) and delayed growth. Mixed infections of BanMMV with BSVs, cucumber mosaic virus (CMV) or BBrMV result in synergistic symptoms that can lead to leaf necrotic lesions (Jones, 2000).

BanMMV is vertically transmitted by vegetative propagation from mother to daughter plants. There is molecular evidence of plant-to-plant transmission (Teycheney, et al., 2005; Diouf et al., 2023) although no biological vector has been identified. The use of virus-free planting material is the only option for the control of BanMMV.

2.7.5. *Cucumber mosaic virus*

Cucumber mosaic virus (CMV) has one of the largest host ranges among plant viruses, with an estimated 1200 host species. It is present worldwide and belongs to the genus *Cucumovirus* in the family *Bromoviridae* (Bujarski et al., 2019).

CMV causes variable symptoms in banana depending on cultivars, plant age and climatic conditions, ranging from sporadic mosaics and line patterns (Figure 12) to severe chlorotic streaks that are very similar to the symptoms caused by BSVs (Kumar et al., 2015). In worst cases, CMV infection causes necrosis of the cigar leaf and heart rot, leading to the death of infected plants. However, in most situations, infected plants recover and symptoms disappear, probably due to natural silencing-based antiviral mechanisms (Zhang et al., 2017).

CMV is transmitted on the non-persistent mode by at least 75 different aphid species and it is vegetatively transmitted through suckers. As for other plant viruses, there are no curative methods against CMV. Prophylaxis, eradication of alternative hosts such as weeds within and around banana plots and using virus-free planting material are efficient to avoid the spread of the disease.

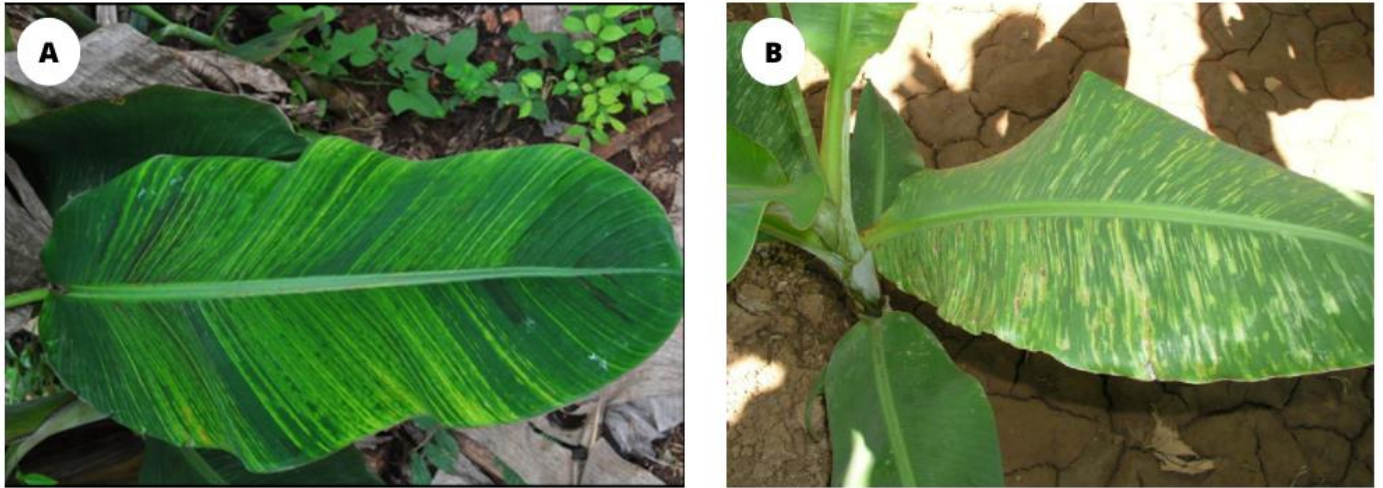


Figure 12: Symptoms of cucumber mosaic virus (CMV) on *Musa spp.*

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2.8. Diagnostic of viruses infecting banana

Virological diagnosis relies on a set of principles, methods and strategies to detect, quantify, monitor and accurately identify viral infections. When several diagnostic tools and methods are available to choose from, the choice depends on performance criteria such as analytical sensitivity & analytical specificity, cost and time required to complete diagnostics (ANSES, 2015). A wide range of immunological, molecular and immuno-molecular tools are available to detect viruses infecting banana (Thomas, 2015). For this study, we used the official diagnostic methods optimised and/or developed and validated the French Agency for Food, Environmental and Occupational Health and Safety (ANSES). ANSES is the French government agency whose main mission is to assess health risks in food, the environment and work. It is accountable to the French Ministries of Health, Agriculture, the Environment, Labour and Consumer Affairs. ANSES is in charge of setting the standards in the diagnostics of plant pests and pathogens (ANSES, 2022).

2.9. Context of the study in La Réunion:

Reunion Island is the most populated and second largest of France's five overseas departments, with a population of 863,100 as of January 1, 2020 (Insee, 2023) and a surface area of 2,512 km², respectively. La Réunion is part of the Mascareignes archipelago and is located in the Indian Ocean, more than 9,000 km from mainland France. It is of volcanic origin, has a tropical climate and more than 100,000 hectares listed as a national park. Some remarkable natural sites of the island are recognised as world heritage by UNESCO (UNESCO, 2013).

Like most other French overseas departments, La Réunion relies on imports to cover its needs for foodstuff since local production does not entirely cover local demand. Two thousand independent producers produce 75% of the domestic needs in fresh products but only 5% of

the needs in processed goods (*La production fruitière à La Réunion*, 2014). Only 17% of the island's surface remains dedicated to agriculture, and this figure keeps decreasing over time due to increasing land pressure exerted by real estate and the impossibility to extend farming to natural habitats that are protected by law. Sugarcane remains the most cultivated crop, using 57% of agricultural land and getting 90% of public subsidies allocated to agriculture. However, this situation is changing, due to incentives to increase the local production of fruits and vegetables in order to increase food autonomy by 2030 (ODEADOM, 2019). Hence farmers are encouraged to shift from sugarcane to other vegetable and fruit productions, such as banana (DAAF La Réunion, 2019).

Banana is grown in private (Creole) gardens for domestic consumption and in larger plots for commercial production. The latter has increased since the 2000's due to an increasing local demand, driving the development of small-scale banana farms producing fruits for local markets and supermarkets. In 2018, 5,150 tons of bananas were produced from 722 plots scattered on 477 ha, which represents 17% of the surface allocated to fruit production (DAAF La Réunion, 2019). Banana has become the third most produced fruit crop in La Réunion, after pineapple and citrus, representing 16% of the overall fruit production. Organic banana remains marginal with 160t produced from 16.8 ha, representing 23% of the total production of organic fruits, making banana the second largest organic crop production in La Réunion after litchi (Agreste, 2017).

Cultivars "Grande Naine", "Banane Mignonne" and "Banane Figue", from the Cavendish group, are the main cultivars grown in La Réunion. Besides banana fruit, the male floral bud called "baba figue" is also consumed in traditional Creole dishes.

Vitroplants are the only form of banana germplasm that can be legally imported into French overseas departments, provided that they have been produced by a company accredited by the French Ministry of Agriculture under strict technical specifications ensuring that they are free of pests and pathogens. Once imported, banana vitroplants are acclimated and weaned in certified facilities. This system has proved efficient for controlling pests and diseases and preventing the importation of the most destructive ones such as TR4 and BBTV in the French overseas departments. However, the use of local suckers as planting material, whose sanitary status cannot be guaranteed, is allowed and probably accounts for the persistence of pests and pathogens such as nematodes, borers and viruses.

2.10. Objectives of the study

Pests and diseases, including viruses, are major constraints worldwide for banana production. Hence their management is instrumental in reducing yield losses, increasing food security and self-sufficiency, and meeting the growing demand for fresh products, especially in landlocked regions and islands such as La Réunion, where food security still relies on imported commodities.

Methods for controlling banana viruses in La Réunion rely on the use of virus-free planting material and epidemiological surveillance of quarantine-regulated viruses (BBTV and BBrMV) in commercial plantations. Both are enforced by the French Ministry of Agriculture. However, nothing is known about the presence, prevalence and diversity of banana viruses in La Réunion, especially among wild banana plants growing along roadsides that are potential reservoirs. The objective of this work is to fill this gap in knowledge.

For this, we carried out an extensive sampling campaign along roadsides and in a few cultivated plots. We optimised immunological, immuno-molecular or molecular virus indexing techniques to allow rapid mass screening of the most prevalent banana viruses (BanMMV, BBrMV, BBTV, BSVs, CMV) in collected samples. We implemented statistical methods to analyse virus prevalence and investigated the molecular diversity of one of the detected virus.

3. Material and methods

Samples were prepared and processed in the plant health laboratory of Anses in Saint-Pierre, La Réunion (<https://www.anses.fr/en/portails/1812/content/152028>).

3.1. Plant materials

Banana leaf samples were collected throughout La Réunion from plots cultivated for commercial production and roadsides. For this, a piece of the youngest attainable expanded leaf was harvested and stored, a labelled paper envelope in a fridge at 4-6°C until further use. Additional samples were collected from CIRAD's banana collection and experimental plots located in Bassin Plat. For each collected sample, the name of cultivar, genotype, GPS coordinates and altitudes of the sampling site, presence of symptoms and mealybugs were recorded (see supp. Table 1 in annexes). Help for identifying accession names and genotypes was obtained from Dr Christophe Jenny (CIRAD, France) and the iNaturalist app (<https://www.inaturalist.org/>) under the "Banana natural biodiversity mapping"¹ project. Some frozen or lyophilized infected and healthy controls were provided by ANSES.

3.2. Sampling design.

Roadside banana plants were randomly sampled along predefined routes. Only banana plants at the flowering or fructifying stage were sampled since pictures of flowers and/or fruit bunches were needed to identify cultivars and genotypes. All 28 plants of CIRAD's banana collection in Bassin-Plat (CIRAD) were sampled. For sampling CIRAD's experimental plot in Bassin-Plat, a systematic sampling was performed as follows: within a rectangle of 11 by 14 banana plants, samples were collected every 3 plants in each row (K=3; Supp Figure 1 in the annexes). GPS coordinates and altitudes were recorded at the four corners of the rectangle.

¹ <https://www.inaturalist.org/projects/banana-natural-biodiversity-mapping>

3.3. Sample pre-processing

Samples were individually processed within five days following harvest, during which they were stored in a fridge at 4-6°C. Each sample, leaf and veins, was individually cut into approximately one-centimetre square pieces, and 0.5 g aliquots (0.1 g when using lyophilised samples) were placed in nylon mesh bags (Bioreba AG, Reinach, Switzerland). When not processed immediately for virus indexing, aliquots were stored at -20°C until further use whereas the remaining leaf pieces were stored at -80°C in paper envelopes.

3.4. Crude extract preparation

Aliquots were ground for 30 seconds in 4.5 ml of either General Extract Buffer (GEB AGDIA Emea, Soisy-sur-Seine, France) or KAJI buffer (De Clerck et al., 2017), using a Homex 6 homogeniser (Bioreba, Reinach, Switzerland). Crude extracts were transferred to 1.5 mL ultracentrifuge tubes and clarified by centrifugation for 5 minutes at 8,000g. Supernatant was used immediately for virus indexing, either undiluted or diluted in DEPC-treated water (Ambion, Thermofisher, Villebon sur Yvette, France) or in crude extract of healthy plants, and the remainder was stored at -20°C until further use.

3.5. Extraction and purification of nucleic acids

Total DNA and total RNA were extracted from leaf material using DNeasy® Plant and RNeasy® Plus mini kits (Qiagen, Courtaboeuf, France). Extractions were performed according to the supplier's protocol except that leaf samples were ground at room temperature. Purified DNA and RNA were stored at -18°C or less until they were used.

3.6. Virus indexings

Indexing protocols used in this study were primarily those validated by ANSES (ANSES, 2017a, 2017b, 2019, 2021). Additional indexing methods were tested. Changes were made to some protocols. For example, diluted plant crude extracts (Massart et al., 2008) were used as a substitute for RNA and DNA extraction and purification to carry out BanMMV and BBTv diagnostics.

Multiplex immunocapture PCR and multiplex RT-PCR were used for the simultaneous detection of four banana streak virus species (BSOLV, BSGFV, BSIMV and BSMYV; (Le Provost et al., 2006; ANSES, 2021) and that of BanMMV, BBTv, BBrMV, CMV and BSOLV (Liu et al., 2012, modified by Maria Roche (pers. comm)), respectively, using either total RNA or crude extracts as templates (Table 4).

All diagnostic methods used or tested during this study are summarised in table 4.

Table 4 : Virus indexing techniques and primers used in this work.

Virus	Diagnostic method	Primer name	Primer sequence	Size of expected amplicon (bp)	References
banana mild mosaic virus (BanMMV)	Polyvalent Degenerate Oligonucleotide RT-PCR (PDO-RT-PCR)	PDO-F1i	TITTYATKAARWSICARYWITGIAC	362	Foissac et al., 2005; Teycheney et al., 2007
		PDO-3Ri	GCRACATRTRCRTCICIGCRAAIIA		
		PDO-4i	ARIYICCATCCRCARAAMITIGG		
		PDO-R1i	TCHCCWGTRAAICKSATIAIIGC		
		PDO-F2i	GCYAARGCIGGICARACIYTKGCITG		
	RT-PCR	BanCP1	GGATCCCGGGTTTTTTTTTTTTTTTTTTT	311	Teycheney et al., 2005
		BanCP2	TATGCNTTYGAYTTCTTRGAYG		
RT-PCR*				Massart, personal communication	
Multiplex RT-PCR	BanMMCP8	TGCCAACTGAYGARGARYTRAAHGC	280	Thomas, JE. 2015.	
	Poty 1	GGATCCCGGGTTTTTTTTTTTTTTTTTTT			
banana bract mosaic virus (BBrMV)	IC-RT-PCR	Bract N1	GGRACATCACAAATTTAATGG	262	Iskra Caruana et al., 2008
		Bract N2	ACATGGAGTATGATGGATAAGG		
	Multiplex RT-PCR	BBrMV-F	CGATACAGAGGGAACCTCTCACCA	132	Liu et al., 2012
		BBrMV-R	GTCTTGAGATGGGCTTCGATACTGTG		
Banana streak OL virus (BSOLV)	Multiplex RT-PCR	BSV-F	GAGACCAAGGTACACAAAATATCATC	1055	Liu et al., 2012
		BSV-R	AACTCTGGTTTTCTTAACCTCTTC		
Banana streak GF virus (BSGFV)	multiplex immunocapture PCR (M-IC-PCR)	OL-R	GCTCACTCCGCATCTTATCAGTC	522	Lagoda et al., 1998; Geering et al., 2000; Le Provost et al., 2006; Geering et al, 2011
		OL-F	ATCTGAAGGTGTGTTGATCAATGC		
GF-R		TCGGTGAATAGTCTGAGTCTTC	476		
GF-F		ACGAACTATCACGACTTGTCAAGC			
Banana streak IM virus (BSIMV)		Im-R	CACCCAGACTTTTCTTTCTAGC	384	
Im-F		TGCCAACGAATACTACATCAAC			
Banana streak MY virus (BSMYV)		MY-R	TAAAAGCACAGCTCAGAACAAACC	589	
		MY-F	CTCCGTGATTTCTTCGTGGTC		
		AGMI025	TTAAAGGTGGGTTAGCATTAGG	248/252*	
		AGMI026	TTTGATGTACAATGGTGTTC		
banana bunchy top virus (BBTV)	PCR	BBTV-F rep			Massé, personal communication
		BBTV-R rep			
	Multiplex RT-PCR	BBTV-F	ATGTGGTATGCTGGATGTTT	747	Liu et al., 2012
		BBTV-R	GTTTCATATTTCCCGCTTTGA		
cucumber mosaic virus (CMV)	ELISA	NA	NA	NA	J.S. Hu et al, 1995

NA: not applicable; *: 248 bp for *M. acuminata*; 252 bp for *M. balbisiana*

3.7. Analysis of amplification products

Amplification products were analysed by capillary electrophoresis using QIAxcel Advance (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Some amplification products were analysed by electrophoresis on agarose gels prepared in 0.5XTBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0). Gels were stained for 15 mn in a 0.0004 ethidium bromide and destained in distilled water for 30 mn and amplification products were visualised and photographed under UV light.

3.8. Cloning, sequencing and phylogenetic analyses

A selection of amplification products was cloned into vector pMini 2.0 using the NEB PCR cloning kit (New England Biolabs, Evry, France) according to the manufacturer's protocol. Recombinant plasmids were introduced by heat shock transformation into NEB 5-alpha competent *E.coli* (New England Biolabs, Evry, France) and transformed cells were plated on LB medium containing ampicillin at 100µg/ML. One to three recombinant plasmids were purified for each cloning experiment using the Qiaprep kit (Qiagen, Courtaboeuf, France) and sequenced by Macrogen (Leipzig, Germany). Phylogenetic analyses were performed using the MEGA 7 package (<https://www.megasoftware.net/>). Multiple sequence alignments and initial phylogenetic reconstructions (neighbour-joining) were performed using the program CLUSTAL_X with randomized bootstrapping evaluation of branching validity (Thompson et al., 1997). Mean diversities, genetic distances (p-distances calculated on amino acid or nucleotide identity) were calculated using MEGA2 (Kumar et al., 2001).

3.9. Statistical analyses

All statistical analyses and positioning of sampling sites were performed out using Rstudio (RStudio, PBC, Boston, MA <http://www.rstudio.com/>). Samples were classified into three groups based on information collected during the survey, sampling sites, genotype and origin of the planting material. The prevalence of each virus was calculated (number of infected samples/number of samples analysed). Contingency tables were computed with the prevalence survey data for cross-referencing the indexing results for each virus within each group. Based on indexing results and prevalence obtained from the survey, an exact binomial test (Bernoulli) was performed to estimate the prevalence of each virus in the banana population.

4. Results

4.1. Sampling

A total of 463 leaf samples was collected across La Réunion (Figure 13). Sampled plants were collected from a wide range of banana varieties or cultivars (Supp Table 1) and from four main types of sampling sites : CIRAD's banana collection and experimental plots, located in Bassin Plat; commercial plots with dessert banana grown for the local market; and roadsides with wild banana whose origin could not be traced and whose variety, cultivar and genotype were determined for most types with the help of Dr C. Jenny (CIRAD, Montpellier, France) based on photographs of the flowers and/or fruit bunches which were upload on the INaturalist app.

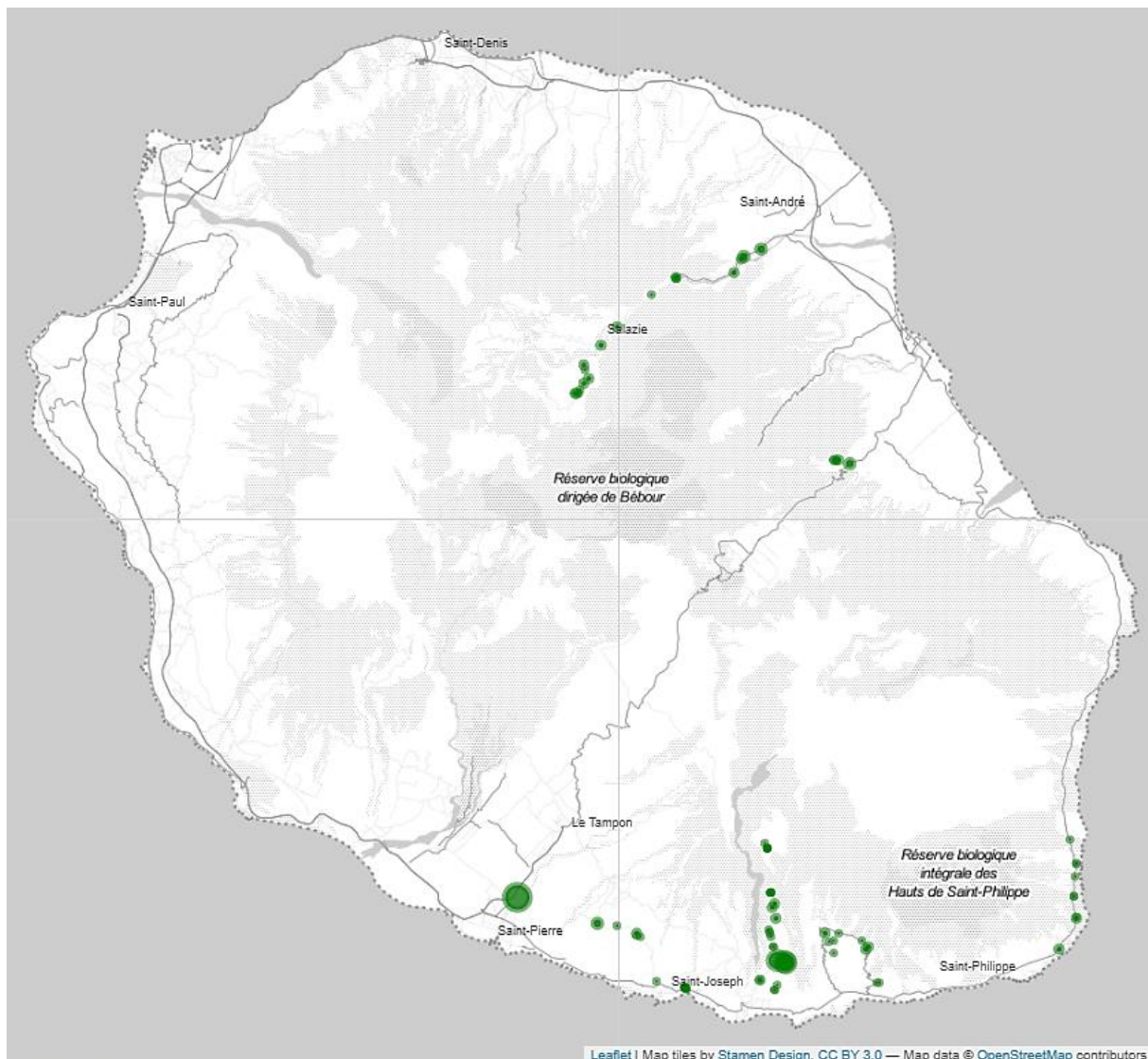


Figure 13: Geographical distribution of collected samples.

Sampling sites are shown with green dots, whose sizes are proportional to the number of collected samples.

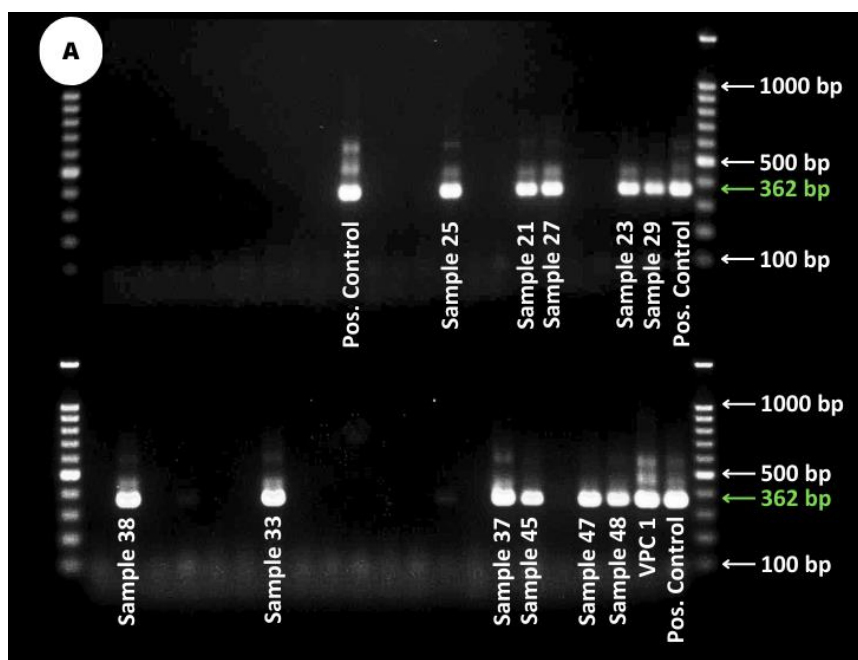
4.2. Assessment of viral indexing methods

The diagnosis of most banana viruses relies on RT-PCR or PCR-based techniques using purified nucleic acids as templates. Processing large amounts of samples can prove tedious and time consuming because of the nucleic acid purification step. Using crude extracts as templates instead of purified nucleic acids (Massart et al., 2008) could save time and cut labour costs substantially when dealing with large amounts of samples such as those collected during this work .

Hence, we assessed the use of plant crude extracts diluted 1:100 as templates for the molecular diagnosis of BanMMV, BBrMV, BBTv, BSOLV and CMV and compared sensitivity thresholds with those obtained using purified nucleic acids as templates. For this, we used samples collected from CIRAD's experimental plot and positive (infected) controls provided by the virology laboratory of ANSES' Unité Ravageurs et agents pathogènes tropicaux (RAPT, La Réunion). All samples indexed positive were indexed a second time to confirm their virological status.

4.2.1. BanMMV

Figure 14 shows the results of comparative analysis for the diagnostic of BanMMV, using the RT-nested-PCR protocol developed by Teycheney *et al.* (2007) based on the PDO primers of Foissac et al. (2005) and optimised by ANSES, and either purified RNAs (A) or crude extracts (B) as templates. Both methods allowed the amplification of a PCR product of the expected size (362 bp) from the positive controls (pos. control and VPC1). A similar amplicon was obtained for 11 samples from CIRAD's collection (# 21, 23, 25, 27, 29, 33, 37, 38, 45, 47, 48) when using purified RNAs as templates (Figure 14 A). When using diluted crude extracts as templates, the same amplicon was obtained from the same samples, except sample #45. We suspect that this difference was due to a pipetting error when loading the gel.



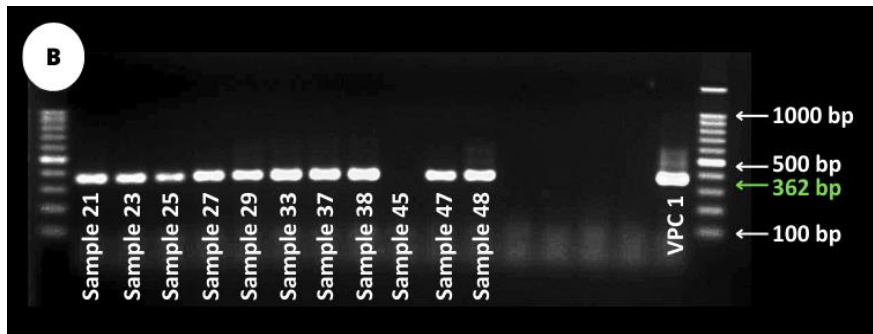


Figure 14: Detection of BanMMV by PDO-RT-nested PCR using purified RNAs (A) or crude extracts diluted 1:100 (B) as templates.

Size of the 500 bp and 1,000 bp fragments of the DNA ladder is shown on the right of the gels. The size of the expected amplification fragment (362 bp) is shown in green.

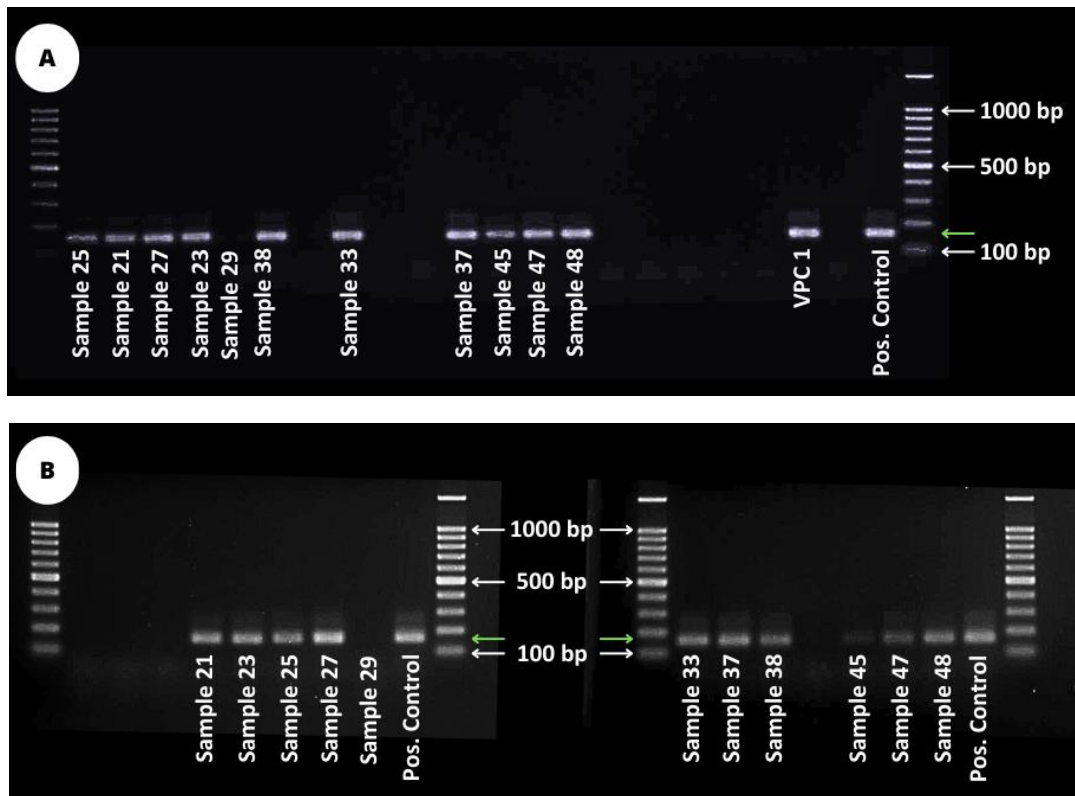


Figure 15 : Detection of BanMMV by RT-PCR using purified RNAs (A) or crude extracts diluted 1:100 (B) as templates.

500 bp and 1,000 bp fragments of the DNA ladder are shown on the gels. The size of the expected amplification fragment is shown with a green arrow.

A similar comparison was performed using a yet unpublished RT-PCR protocol and primer set developed by Massart et al. (pers. comm.) targeting the RNA-dependent RNA polymerase (RdRp) domain of the polyprotein encoded by BanMMV ORF1. For this, the eleven samples from CIRAD's collection that were detected positive for BanMMV using the PDO primer set

(Figure 14) were used together with six healthy samples as negative controls. Figure 15 shows that similar results were obtained using either purified RNAs (A) or crude extracts (B) as templates. Ten of the eleven samples that tested positive with the PDO primer set (#21, 23, 25, 27, 33, 37, 38, 45, 47, 48) were also tested positive with the primers developed by Massart et al., but not sample #29. This result tends to show that the primer pair and experimental protocol developed by Massart et al. might be less inclusive than the PDO RT-nested PCR protocol. However, considering that the former provides a substantial gain in time and budget because it can be performed in a single tube and does not require a nested PCR step, we decided to use it to screen the 443 samples collected during this study.

We then assessed whether the extraction buffers used to grind leaf samples and prepare crude extracts influenced detection thresholds (analytical sensitivity). For this, we used serial dilutions of crude extracts prepared from BanMMV-infected sample #27 and from positive control VPC1 in buffer KAJI or GEB in RT-PCRs performed according to Massart et al. (unpublished). Amplification products were analysed using QIAxcel Advance because it provides an accurate estimate of amplicon's size (Figure 15). This comparison did not provide a definitive conclusion: the detection threshold was apparently lower for sample 27 prepared in buffer KAJI than in buffer GEB, whereas it was the opposite for sample VPC1. Besides, we compared the analytical sensitivity of crude extracts to purified RNAs. For this, we used serial dilutions of purified RNAs prepared from the infected sample BanMMV #27. The detection threshold is higher for purified RNAs than for crude extracts.

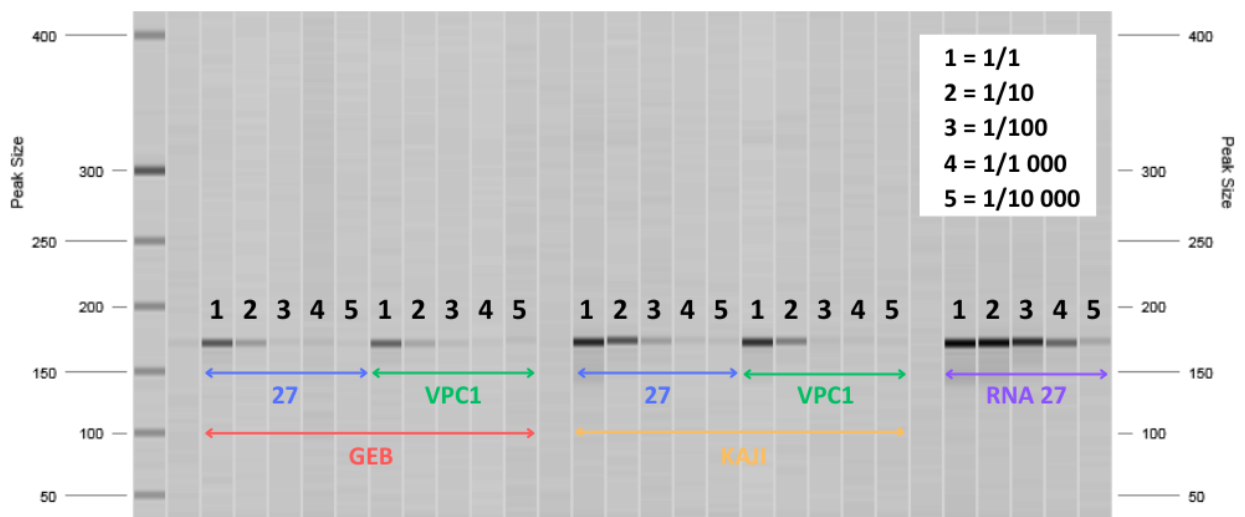


Figure 16: Detection of BanMMV by RT-PCR from crude extracts of samples #27 and positive control VPC1 prepared in buffer GEB or KAJI.

Lane numbers refer to the dilution of crude extracts, which is provided in the caption on the top right corner of the figure. VPC1 purified RNA was used as a control.

4.2.2. BBTV

Two primer sets are available for the detection of BBTV by PCR: primer pair BBTV-F rep / BBTV-R rep was developed by Massé et al. (unpublished) whereas primer pair BBTV-F / BBTV-R was

developed by Liu et al. (2012; table 4). Both primer pairs target a region of BBTV DNA-R encoding the Rep protein. We compared the sensitivity of both primer pairs for the detection of BBTV, using serial dilutions of crude extracts from two positive controls (BBTV 017 and BBTV D22) as templates. We used BBTV purified DNA as a positive control of the PCR experiments. Figure 17 shows that both primer pairs allow the accurate detection of BBTV and that using primer pair BBTV-F rep / BBTV-R rep results in a more sensitive detection of BBTV. Therefore, primer pair BBTV-F rep / BBTV-R was used for indexing the 443 samples of this study.

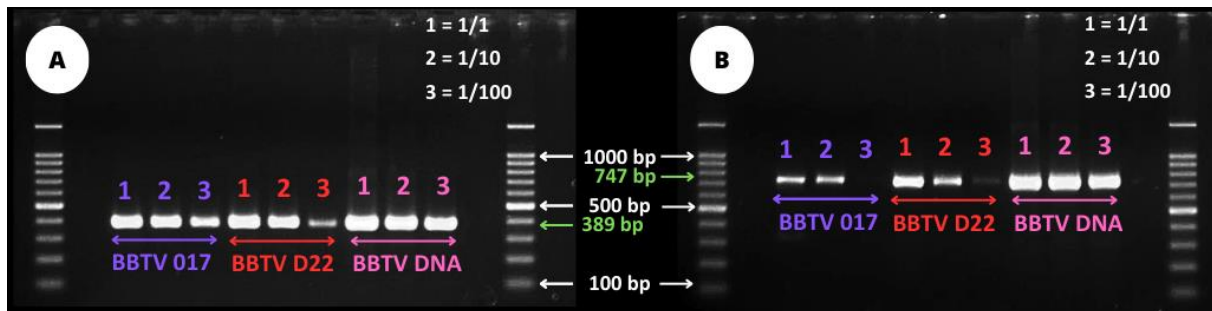


Figure 17: Detection of BBTV by PCR using serial dilutions of crude extracts from infected controls #BBTV 017 and #BBTV D22 as templates, primer pairs BBTV-F rep/BBTV-R rep (A) and BBTV-F/BBTV-R (B).

500 bp and 1,000 bp fragments of the DNA ladder are shown on the right side of the gel. The size of the expected amplification products is shown with a green arrow.

Here again, we assessed whether the extraction buffers used to grind leaf samples and prepare crude extracts influenced detection thresholds. For this, we used serial dilutions of crude extracts prepared from BBTV-infected controls D22 and O17 in buffer GEB or buffer KAJI. Detection thresholds were similar for control #D22 and better when using buffer GEB for control O17 (Figure 15). Once again, we compared the analytical sensitivity of crude extracts to purified DNAs. For this, we used serial dilutions of purified DNA prepared from the infected sample BBTV #D22. Once more, the detection threshold is higher for purified DNA than for crude extracts.

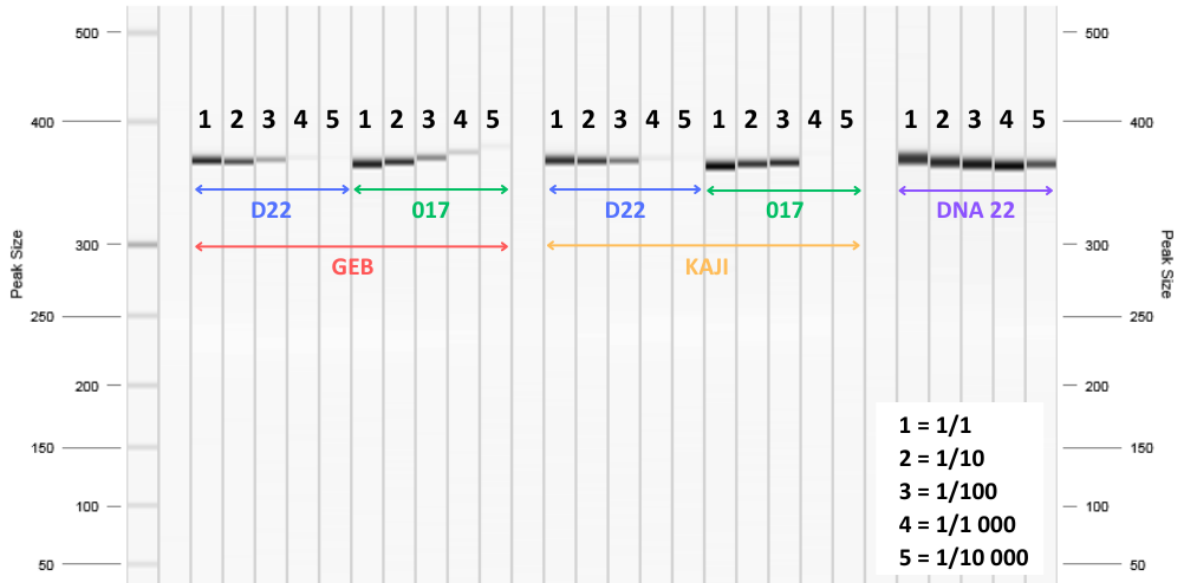


Figure 18: Detection of BBTV by PCR from crude extracts of two positive controls #BBTV D22 and #BBTV 017 prepared in buffer GEB and KAJI.

Lane numbers refer to the dilution of crude extracts, which is provided in the caption on the bottom right corner of the figure. Purified DNA was used as a control.

4.2.3. Multiplex-RT-PCR

A quadruplex RT-PCR method was developed by Liu et al. (2012) for the simultaneous detection of BBrMV, BBTV, BSOLV and CMV. This protocol was modified by M. Roche (ANSES La Réunion, unpublished) to include primer pair BanMMV CP2 / Poty1 developed by J. Thomas (2015) for the detection of BanMMV. We assessed the sensitivity of this modified multiplex RT-PCR for the simultaneous detection of BanMMV, BBrMV, BBTV, BSOLV and CMV using either purified RNAs or crude extracts from infected and healthy plants as templates. Figure 17 shows that BanMMV was not detected by the multiplex assay when using crude extracts as templates for infected samples #, 27, 33, 37, 38 and 47, whereas it was detected in these samples when using purified RNAs as templates. Moreover, a BSOLV-specific amplification product was obtained from samples 27, 29, 33 when using purified RNAs as templates, but not when using crude extracts, whereas it was the opposite for sample #48. It is likely that these amplification products arose from endogenous banana streak viruses (eBSVs; (Chabannes et al., 2013), since these samples originate from Bluggoe or Prata Ana accessions, whose ABB or AAB genomes are known to host eBSVs (Duroy et al., 2016). BBTV was accurately detected from either purified RNAs and crude extracts whereas BBrMV and CMV were detected only from purified RNAs but not from crude extracts.

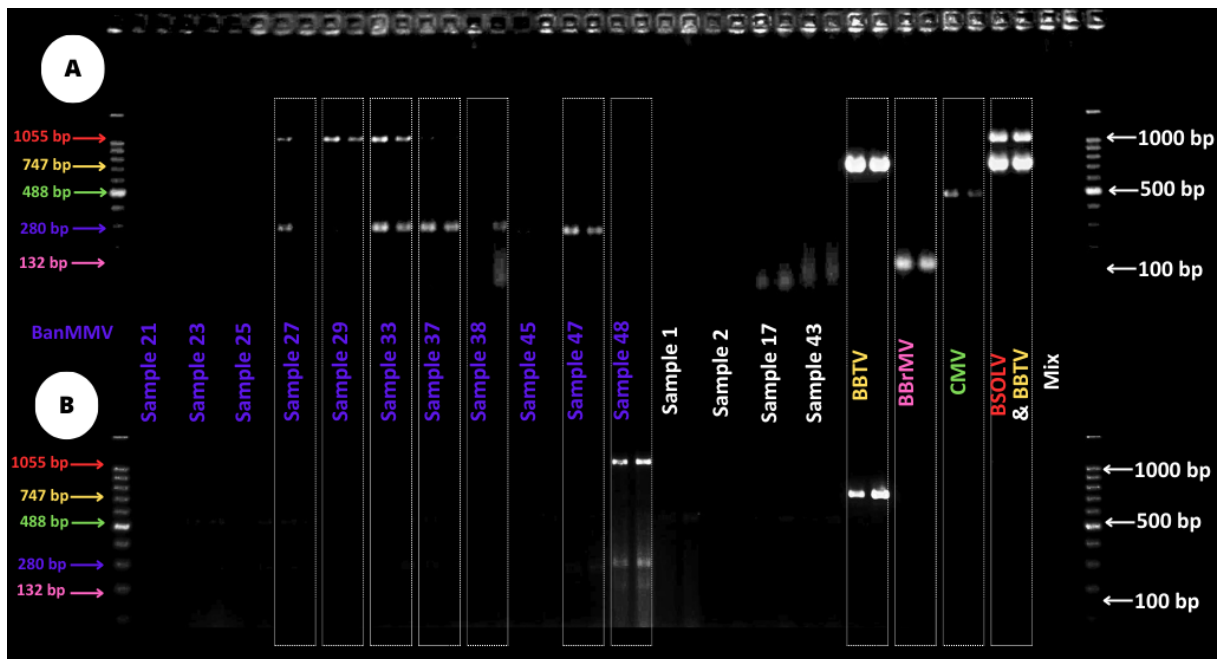


Figure 19: Detection of BanMMV, BBrMV, BBTv, BSOLV and CMV by multiplex RT-PCR using a modified version of the assay developed by Liu et al. (2012) and either purified RNA (A) or crude extracts diluted 1:100 (B) as templates.

Samples #1, 2, 17, 21, 23, 25, 27, 29, 33, 38, 43, 45, 47 and 48 originated from the sampling performed in this work (Supp Table 1). Positive controls for BBTv, BBrMV, CMV and BSOLV were provided by ANSES. 100 bp, 500 bp and 1,000 bp fragments of the DNA ladder are shown on both sides of the gels. The size of the expected amplification products for each targeted virus is shown with coloured arrows on the left side of the gel. Each amplification product was loaded twice in adjacent wells. Infected samples are shown in boxes.

4.2.4. Conclusion

Overall, based on the comparisons of detection methods and thresholds (Figures 14-17), we decided to use crude extracts prepared in buffer GEB for the detection of BanMMV, BBrMV, BBTv and CMV. In addition, we used multiplex-immunocapture-PCR (M-IC-PCR) for the detection of BSOLV, BSGFV, BSMYV and BSIMV in order to avoid false positives resulting from the amplification of eBSVs from interspecific hybrids containing the B genome (Le Provost et al., 2006), IC-RT-PCR for that of BBrMV (Iskra Caruana et al., 2008), ELISA for CMV, PCR for BBTv (Massé, personal communication) and RT-PCR for BanMMV (Massart, personal communication, optimised by N. Cassam (ANSES; unpublished)).

4.3. Prevalence of BanMMV, BBrMV, BBTv, BSOLV, BSGFV, BSIMV, BSMYV and CMV in La Réunion

Tables 5 and 6 summarise the results of the prevalence survey. They show that BanMMV, BSGFV, BSMYV and BSOLV were detected in collected samples, whereas BBTv, BBrMV, BSIMV and CMV were not. Four samples collected from cultivars Bluggoe (ABB genotype) were co-infected by two viruses or more: samples 21, 23 and 25 were co-infected by BanMMV and BSMYV whereas sample 38 was co-infected by BanMMV, BSOLV and BSMYV (Table 5).

Table 5: Details of the samples indexed positive

Sample number	Date sampled	Cultivar	Genotype	Location	Type of plot	Latitude	Longitude	Altitude	BanMMV	BBrMV	BBTV	BSOLV	BSGFV	BSMYV
21	14/2/2023	Bluggoe	ABB	Saint Pierre	Collection	-21322142	55489926	153	+	-	-	-	-	+
23	14/2/2023	Bluggoe	ABB	Saint Pierre	Collection	-21322142	55489926	153	+	-	-	-	-	+
25	14/2/2023	Bluggoe	ABB	Saint Pierre	Collection	-21322142	55489926	153	+	-	-	-	-	+
27	14/2/2023	Bluggoe	ABB	Saint Pierre	Collection	-21322142	55489926	153	+	-	-	-	-	-
29	14/2/2023	prata anna	AAB	Saint Pierre	Collection	-21322142	55489926	153	+	-	-	-	-	-
33	14/2/2023	Bluggoe	ABB	Saint Pierre	Collection	-21322142	55489926	153	+	-	-	-	-	-
37	14/2/2023	Bluggoe	ABB	Saint Pierre	Collection	-21322142	55489926	153	+	-	-	-	-	-
38	14/2/2023	Bluggoe	ABB	Saint Pierre	Collection	-21322142	55489926	153	+	-	-	+	-	+
39	14/2/2023	plantain STOP	AAB	Saint Pierre	Collection	-21322142	55489926	153	-	-	-	-	+	-
40	14/2/2023	plantain STOP	AAB	Saint Pierre	Collection	-21322142	55489926	153	-	-	-	-	+	-
45	14/2/2023	Plantain Bernard	AAB	Saint Pierre	Collection	-21322142	55489926	153	+	-	-	-	-	-
47	14/2/2023	Plantain Bernard	AAB	Saint Pierre	Collection	-21322142	55489926	153	+	-	-	-	-	-
48	14/2/2023	prata anna	AAB	Saint Pierre	Collection	-21322142	55489926	153	+	-	-	-	-	-
126	29/3/2023	Bluggoe	ABB	Saint-Benoit	Roadside	-21096603	55675421	468	-	-	-	-	-	+
127	29/3/2023	Bluggoe	ABB	Saint-Benoit	Roadside	-21096603	55675421	468	-	-	-	-	-	+
132	29/3/2023	Unknown	Unknown	Saint-Benoit	Roadside	-21094882	55669160	483	-	-	-	-	-	+
134	29/3/2023	Unknown	Unknown	Saint-Benoit	Roadside	-21094882	55669160	483	-	-	-	-	-	+
145	29/3/2023	Pome-Prata	AAB	Salazie	Roadside	-21008782	55564740	438	-	-	-	-	-	+
205	19/4/2023	Unknown	Unknown	Saint-Joseph	Roadside	-21369171	55584030	102	-	-	-	-	+	-
206	19/4/2023	Musa X paradisiaca	AAB	Saint-Joseph	Roadside	-21369165	55583800	102	-	-	-	-	+	-
207	19/4/2023	Unknown	Unknown	Saint-Joseph	Roadside	-21369118	55583753	102	-	-	-	-	+	-
208	19/4/2023	Unknown	Unknown	Saint-Joseph	Roadside	-21369140	55583762	102	-	-	-	-	+	-
218	19/4/2023	Unknown	Unknown	Saint-Joseph	Roadside	-21319676	55631152	861	+	-	-	-	-	-
225	19/4/2023	Unknown	Unknown	Saint-Joseph	Roadside	-21296423	55629190	1116	-	-	-	+	-	-
274	26/4/2023	Musa X paradisiaca	AAB	Saint-Joseph	Roadside	-21340364	55661702	569	-	-	-	-	-	+
301	26/4/2023	Pome-Prata	AAB	Saint-Philippe	Roadside	-21332529	55801754	68	-	-	-	-	+	-

Table 6 Results of the prevalence survey

(A) The number and percentage of infected samples is shown for each category of sampling site.

Sampling site	Number of analyzed samples	BanMMV		BSGFV		BSMYV		BSOLV	
		number of infected samples	% infected samples	number of infected samples	% infected samples	number of infected samples	% infected samples	number of infected samples	% infected samples
CIRAD's collection	28	11	39,29	2	7,14	4	14,29	1	3,57
CIRAD's experimental plots	71	0	0,00	0	0,00	0	0,00	0	0,00
Commercial plots	153	0	0,00	0	0,00	0	0,00	0	0,00
Roadsides	191	1	0,52	5	2,62	6	3,14	1	0,52
Total	443	12	2,71	7	1,58	10	2,26	2	0,45

(B) The number and percentage of infected samples is shown for each category of sampled genotype.

Genotype	Number of analyzed samples	BanMMV		BSGFV		BSMYV		BSOLV	
		number of infected samples	% infected samples	number of infected samples	% infected samples	number of infected samples	% infected samples	number of infected samples	% infected samples
AAA	235	0	0,00	0	0,00	0	0,00	0	0,00
AAB	36	4	11,11	3	8,33	1	2,78	0	0,00
ABB	24	7	29,17	0	0,00	6	25,00	1	4,17
Unknown	148	1	0,68	4	2,70	3	2,03	1	0,68
Total	443	12	2,71	7	1,58	10	2,26	2	0,45

4.3.1. BanMMV

Twelve samples were tested positive for BanMMV: 11 samples originated from accessions collected in CIRAD's collection in Bassin Plat (# 21, 23, 25, 27, 33, 36, 37, 38, 39, 45, 46, 47) whereas one sample (#218) originated from a sample collected on a roadside in St Joseph (Table 5). The virological status of all infected plants was confirmed by RT-PCR performed on purified RNAs using either the PDO primer set or primer pair BanCP1/BanCP2, which targets a 210 nt region of the BanMMV genome encompassing the end of ORF5 encoding the coat protein and the 3' untranslated region (3'UTR) (Teycheney et al., 2005; data not shown). The prevalence of BanMMV was high in CIRAD's collection (39.3%; 11/28), which originates from non-certified vegetatively propagated planting material (suckers) produced from plants collected throughout La Réunion (D. Carval, pers. com.). None of the 19 samples collected on the same plots from plantain K74 plants (samples #101-119, Supp. Table 1) were indexed positives. These plants originate from vitroplants.

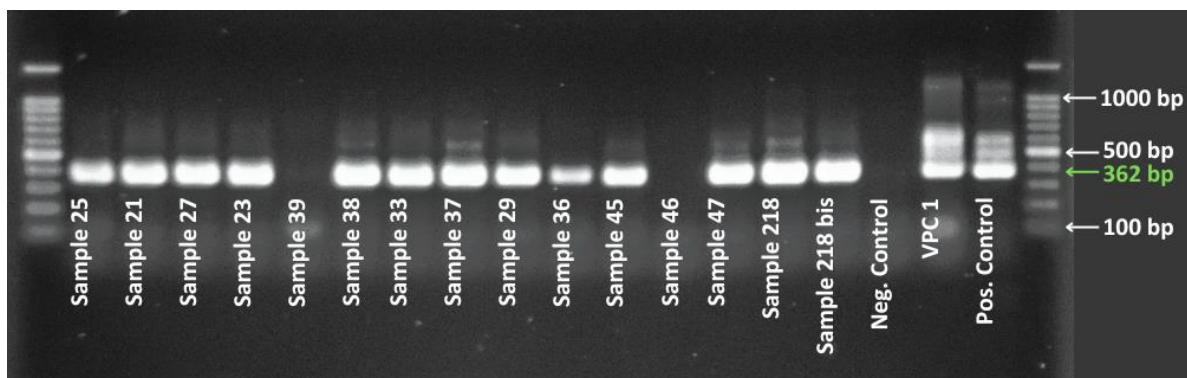


Figure 20: Confirmation of the virological status of BanMMV-infected samples using the PDO-RT-nested PCR approach on purified RNAs.

500 bp and 1,000 bp fragments of the DNA ladder are shown on both sides of the gel. The size of the expected amplification products (362 bp) is shown with coloured arrows on the left side of the gel.

4.3.2. BSVs

Eighteen samples were indexed positive for at least one BSV species (Table 9). Six of them originated from CIRAD's collection: three were infected by BSMYV, two by BSGFV and one was co-infected by BSMYV and BSOLV. The remaining twelve infected samples originated from roadsides: six were infected by BSMYV, five by BSGFV and one by BSOLV. Although the overall prevalence of BSVs in our sampling was low (3.9%, 18/463), it was high in CIRAD's collection (21.4%; 6/28). All BSV-infected accessions whose genome could be identified included the B genome and belonged to the Bluggoe group (ABB genotype; 6/18), Prata Ana (AAB; 2/18), *Musa x paradisiaca* (AAB; 2/18) or plantain (AAB; 2/18).

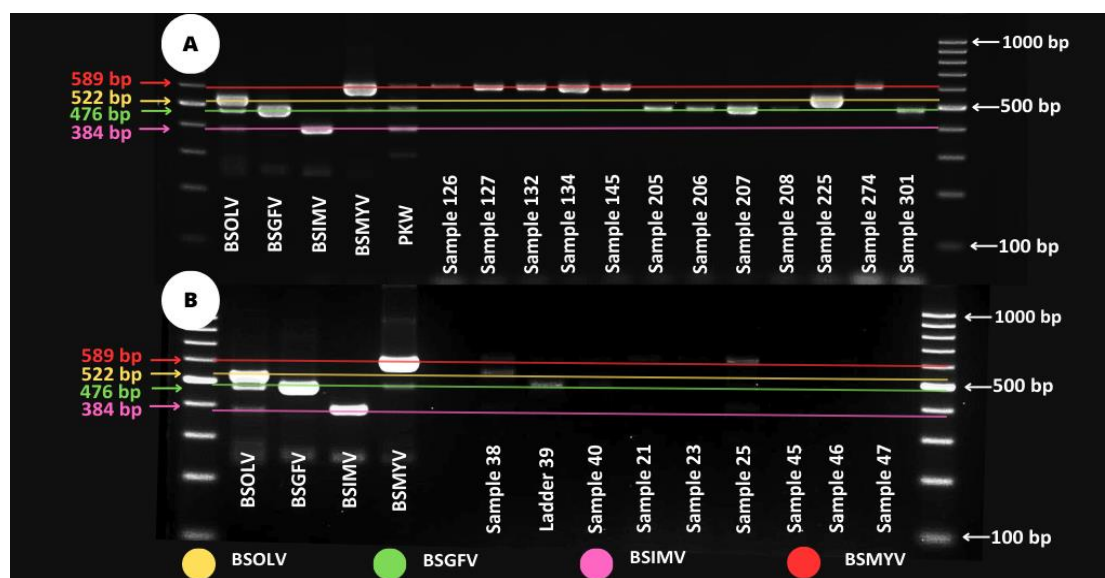


Figure 21: Detection of BSOLV, BSGFV, BSIMV and BSMYV by M-IC-PCR.

100 bp, 500 bp and 1,000 bp fragments of the DNA ladder are shown on the right side of the gels. The sizes of the expected amplification products are shown with coloured arrows on the left side of the gels. The color codes to BSV species is shown at the bottom of the figure.

4.4. Summary of the results and statistical analyses

All 443 leaf samples were indexed for BanMMV, BBTv, BBrMV, CMV, BSGFV, BSMYV, BSIMV and BSOLV. The prevalence of each virus (percentage of infected plants), were calculated for each sampling site and genotype (Tables 6 A and 6 B). Overall prevalence was low for all detected viruses: 2.71% for BanMMV, 1.58% for BSGFV, 2.26% for BSMYV and 0.45% for BSOLV (Table 6A). All detected viruses were more prevalent in CIRAD's *Musa* collection than in any other sampling sites (Table 6 A).

Prevalence was also higher in accession with B-containing genomes (Table 6 B). BSGFV infections occurring in AAB genotypes may result from the activation of infectious eBSVs, as observed in Guadeloupe during a similar study (Umbert et al., 2022). The genotype of 148 samples could not be identified based on photographs of inflorescences and/or fruit bunches. Prevalence within this group was as follows: BanMMV: 0.68%; BSGFV: 2.70%; BSMYV: 2.03%; BSOLV: 0.68%.

No virus was detected from samples with AAA genotypes, which were mostly collected from cultivated plots and originated from certified vitroplants.

The heterogeneous size of the groups defined on the basis of sampling sites or genotypes, and the overall low number of infected samples precluded accurate data statistical analyses. Therefore, no correlation could be established between virus prevalence and the various parameters collected during sampling. Similarly, the results of the binomial test carried out to estimate the prevalence of each virus within populations may be statistically biased by several factors such as reduced sample counts per category of sample and limited representativeness or over-representation in samples collected from CIRAD's germplasm collection.

4.5. Molecular diversity of BanMMV

A previous study showed that BanMMV isolates from Guadeloupe display a high molecular variability. Mean pairwise nucleotide sequence divergence levels of more than 20% were registered among these isolates in a region of the viral genome encoding conserved motifs II and V near the active site of the RdRp (Teycheney et al., 2005)). We investigated the molecular variability of the BanMMV isolates detected during this study and compared it to that of isolates from Guadeloupe and the rest of the world. For this, we cloned and sequenced the amplification products obtained from BanMMV-infected accessions either by PDO-RT-PCR using the PDO primer set or by RT-PCR using primer pair BanCP1/BanCP2 (Table 4). Phylogenetic analyses of the sequences were performed by Thierry Candresse (INRAe, UMR BFP, Villenave d'Ornon, France).

Neighbour joining trees built from phylogenetic analyses performed on the RdRp (Fig. 22 A) and CP/3'NCR (Fig. 22 B) showed that the overall diversity of the BanMMV isolates from the La Réunion datasets displayed a lower molecular diversity than those from Guadeloupe and/or

the rest of the world. This was confirmed by the analysis of the mean diversities within each dataset (Table 7). Considering that the number of sequences in the La Réunion dataset (33) was significantly lower than that of the Guadeloupe (146) and RoW (55) datasets and that this difference might have introduced a bias in the analysis, the mean diversity was also calculated five times independently on 33 randomly selected sequences from the Guadeloupe dataset (146 sequences; Table 7). The figure we obtained from this dataset (20.2% +/- 1.2%) was very similar to the figure obtained from the complete Guadeloupe dataset, providing a strong indication that the difference observed between the La Réunion and Guadeloupe datasets was not biased by the smaller size of the La Réunion dataset.

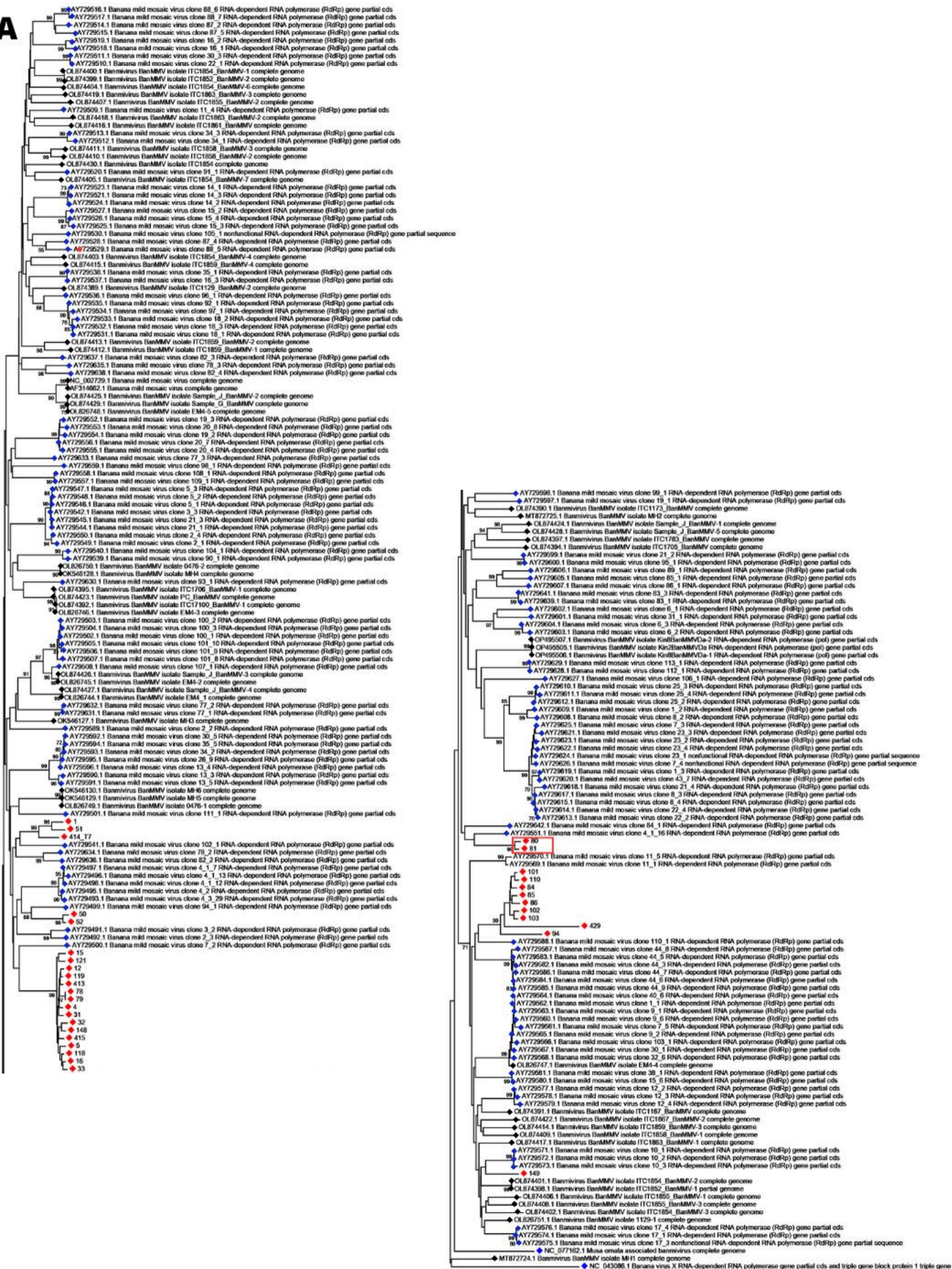
A similar comparison could not be carried out using the CP/NCR dataset because this dataset did not contain enough sequences (7).

Table 7: Mean nucleotide diversities for the BanMMV RdRp dataset

	Number of sequences	Mean diversity	Standard deviation
La Réunion	33	14,7%	1,0%
Guadeloupe	146	20,5%	1,3%
Subset Guadeloupe*	33	20.2%	1.2%
RoW (rest of the world)	55	19,0%	1,1%
Guadeloupe + RoW	203	20,30%	1,1%

**: mean diversity calculated from 5 independent repeats performed on 33 randomly selected sequences from the Guadeloupe dataset (146 sequences).*

A



0.02

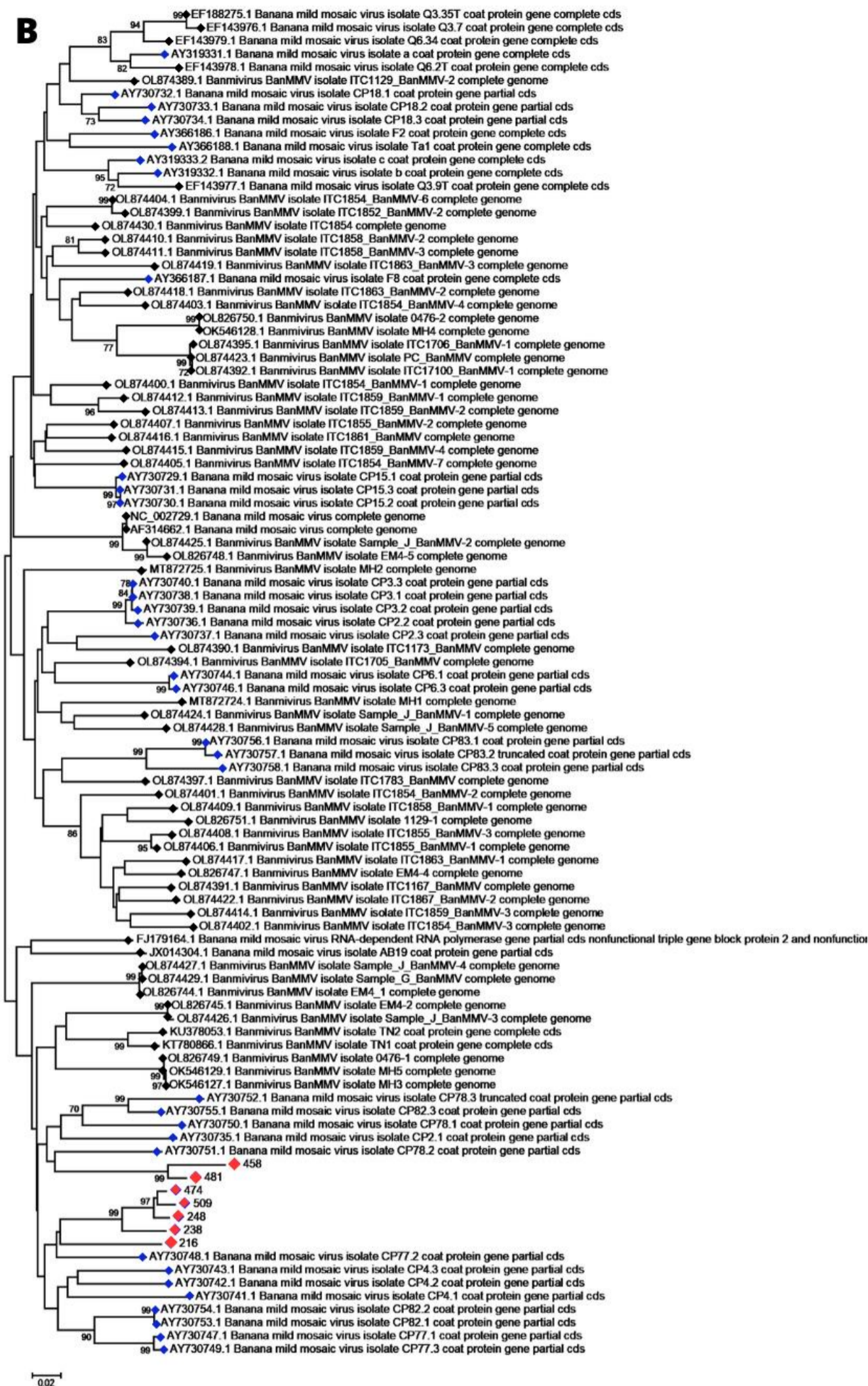


Figure 22: Neighbour joining phylogenetic trees showing the relationships between BanMMV isolates.

Phylogenetic analyses were performed on a 362 bp sequence encoding conserved motifs II and V near the active site of the RNA-dependent RNA polymerase (A) and a 320 bp sequence covering the last 234 nt of the coat protein gene and the complete non-coding region. Nucleotide sequence alignments were generated using Clustal and phylogenetic analyses were done using IQTree v. 1.7 beta with the HKY model. Support values above 95% from UltraFast bootstrap method with 10,000 replicates are shown above nodes. Banana virus X (unassigned genus, family *Betaflexiviridae*) and *Musa ornata*-associated banmivirus were used as outgroups. Red, blue and black diamonds indicate sequences originating from La Réunion, Guadeloupe and the rest of the world, respectively. Sample numbers from which sequences generated in this work originated are provided (1, #25: sequence number 1 originating from sample number 25, e.g)

5. Discussion

Prior to this study, little was known about the prevalence and diversity of viruses infecting banana in La Réunion. The main objective of this work was to address this knowledge gap. For this, we collected banana leaf samples from three types of locations corresponding to the three types of plants we aimed to address: commercial plantations with plants originating from certified *in vitro* plants, CIRAD's germplasm collection with plants originating from non-certified suckers collected throughout La Réunion and roadsides with wild banana plants of unknown origin, respectively. We purposely collected a number of samples (443) that would be manageable considering the duration of my internship (24 weeks).

Selecting indexing methods that meet performance criteria such as sensitivity and specificity was critical to the success of this work, since the time required to collect, process and index samples would determine the number of samples that could be analysed. A large array of immunological, immuno-molecular, and molecular indexing techniques was available to choose from for detecting the targeted viruses (BanMMV, BBrMV, BBTv, BSVs and CMV). Different methods were compared, considering templates (plant crude extract, purified nucleic acids), grinding buffers, primer sets and PCR-based methods.

Initially, a multiplex RT-PCR approach was considered for the simultaneous detection of all viruses targeted by our study. However, this method proved ineffective when using either crude extracts or purified nucleic acids as a template: only BBTv could be detected when using crude extracts, whereas this technique lacked sensitivity when using purified nucleic acids, resulting in false negatives. Moreover, this multiplex assay did not allow the detection of BSGFV, BSMYV and BSIMV and did not discriminate between episomal and endogenous BSOLVs in accessions with B-containing genomes, resulting in false positives. Hence, this multiplex assay was not retained.

For the diagnosis of BanMMV, the comparison of the PDO-RT-nested-PCR protocol of Teycheney et al., (2007) optimised by ANSES (unpublished) using the degenerate primers developed by Foissac et al. (2005) and the RT-PCR protocol of Massart et al. (pers. comm.) optimised by N. Cassam (ANSES; unpublished) showed that both methods raised similar results using either crude extracts or purified nucleic acids as templates, except for sample #29, which was not indexed positively by the method of Massart et al. This result suggests that the primer set developed by Massart et al. might be less inclusive than the highly degenerated PDO primers of Foissac et al. (2005), which allowed the detection of highly diverse BanMMV isolates (Teycheney et al., 2005a) and even that of a distinct and yet unassigned member of the family *Betaflexiviridae*, banana virus X (Teycheney et al., 2005b; Teycheney et al., 2007). The time saved using the single tube detection method designed by Massart et al. optimised by N. Cassam (ANSES; unpublished) compared to the 2 step PDO-nested-RT-PCR protocol of Teycheney et al (2007) was weighed against the loss of sensitivity. It was deemed more beneficial to take the risk of false negatives, therefore it was decided to retain the method of Massart et al. using crude extracts as template for our prevalence study.

Similarly, two protocols for the detection of BBTv, based on different primer pairs, BBTv-F rep /d BBTv-R rep (Massé et al., unpublished) and BBTv-F /BBTv-R (Liu et al. (2012), were compared. Again, this comparative analysis showed that both protocols raised similar results

using either crude extracts or purified nucleic acids as templates. However, the primers developed by Massé et al. enabled a more sensitive detection in the samples that we tested, therefore these primers were selected for our prevalence study, using crude extracts as templates.

Lastly, a comparative analysis was carried out to assess the influence of the extraction buffer on the sensitivity of virus indexing. We found that detection thresholds were similar for the detection of BanMMV and BBTV using either KAJI buffer or GEB buffer for the sample grinding step. GEB buffer was therefore suitable for the detection of all viruses targeted by this study. This finding made it possible to use a single sample preparation method and buffer (GEB) for virus testing and save a considerable amount of time during sample processing. Therefore, the GEB buffer was selected over KAJI for our prevalence study.

Additionally, a QIAxcel Advance capillary electrophoresis instrument (Qiagen) was chosen to analyse the amplification products because it allows for the rapid analysis of samples using 96-well plates, without manual intervention and with minimal manipulation. This reduces handling errors and eliminates gel preparation, sample loading, and exposure to ethidium bromide.

In summary, we opted for the detection of BanMMV by RT-PCR, using the unpublished protocol of Massart et al., BBrMV by IC-RT-PCR using the protocol of Iskra Caruana et al. (2008), BBTV by PCR using the protocol of ANSES, 2019, episomal BSOLV, BSGFV, BSMYV and BSIMV by M-IC-PCR, using the protocol of Le Provost et al. (2006) modified by Umber *et al.* (2016) and CMV by ELISA. We did not carry out comparative analysis nor modified the indexing protocols for BBrMV, BSVs, and CMV, which were optimised and validated by ANSES prior to this work and are currently the official diagnostic methods for the detection of these viruses used by plant protection organisations in France. All selected indexing techniques were performed on the same crude extracts prepared in the GEB buffer.

However, the methodological improvements made during this study were only conducted once, and performance criteria such as repeatability and reproducibility were not assessed. Although, the results of our study indicate that these methodological improvements seem promising, before they can be used more reliably, additional trials and validations need to be considered.

Our prevalence survey led to several significant findings. Firstly, BBTV, BBrMV, BSIMV and CMV were not detected in the analysed samples. The absence of BBTV and BBrMV, which are quarantine regulated pests, confirmed the findings of FDGDON, which has been in charge of the epidemiological surveillance of both viruses in commercial plots in La Réunion for years and has never detected these viruses. The absence of CMV in the analysed samples was more surprising, considering that this virus is highly prevalent in many crops worldwide, including La Réunion, and that many widespread weeds are natural reservoirs of this virus. We assume that the absence of cultivated plots with crops known to host CMV, such as solanaceous crops (tomato, bell pepper, cucurbits e.g), near sampling sites results in a low or non-existent circulation of CMV in/near these sites. Our results also suggest that BSIMV is either absent or present at a low prevalence in La Réunion. A similar situation was reported previously from several Caribbean islands (Javer Higginson et al., 2014; Martinez et al., 2016; Umber et al., 2022). BanMMV, BSGFV, BSMYV and BSOLV were detected in our samples. It was the first

detection of BanMMV in La Réunion, and this result will be published as a Disease Note in *Plant Disease*. The overall prevalence of BanMMV, BSGFV, BSMYV and BSOLV was low (0.45% to 2.71%) and no symptom could be observed on infected plants, pointing to a negligible impact of these viruses on infected plants. This situation mimics previous findings from similar studies carried out in Guadeloupe, where prevalence of BanMMV was also low (Perefarres et al., 2007) and the impact of BSVs and BanMMV was negligible (Perefarres et al., 2007; Umber et al., 2022). We found that the prevalence of BanMMV, BSGFV and BSMYV varied between sampling sites and/or sample genotypes. For example, no virus was detected in the samples collected from cultivated plots, which were planted 2-5 years ago with certified vitro plants. This observation suggests that the dynamics of viral (re)contamination is low and confirms that the use of certified planting material prevents the spread of viral diseases in bananas. Conversely, most BSGFV- and BSMYV-infected samples originated from samples collected from roadsides and from CIRAD's germplasm collection, i.e from naturally propagated and/or non-certified planting material (suckers). These results further confirm the effectiveness of using certified planting material for controlling the spread of banana viruses and the potential role of vegetatively propagated banana plants in the epidemiology of banana viruses. Interestingly, all BanMMV-infected samples are originated from CIRAD's germplasm collection, whose accessions originate from non-certified suckers, whereas BanMMV was not detected from any of the indexed K74 plants planted on the same plot, which originate from in vitro plants and were planted at the same time as suckers, in June 2021. This finding suggests that the observed BanMMV infections are more likely to result from vertical transmission (from mother plant to daughter plant) than from vector-borne (horizontal) transmission. It is worth noting that although there is indirect evidence for plant-to-plant transmission of BanMMV (Teycheney et al., 2005), no vector has yet been identified.

We found that the molecular diversity of the BanMMV isolates from La Réunion was significantly lower than that of isolates from Guadeloupe or the rest of the world. Interestingly, sequences from an isolate (#29) that was indexed positive using highly degenerate primers but negative using the primers of Massart et al. (unpublished) were highly similar but significantly different from the sequences amplified from the other BanMMV isolates from La Réunion. Further sequencing data will be generated and whole-genome analyses will be carried out to find out if these differences prevent the annealing of one of the primers or both primers developed by Massart et al.

Overall, this first large-scale study of banana viruses in La Réunion provides useful information on the prevalence and diversity of banana viruses in La Reunion, although it has limitations due to the relatively small size of our sampling. The methodological improvements made during this study require additional testing and validation to reliably ensure that they meet performance criteria such as sensitivity, specificity, repeatability, reproducibility, cost- and labour-efficiency. However, these methodological improvements seem promising and could pave the way for future larger scale studies that could help unravel the epidemiology of banana viruses in La Réunion, including the search for potential contamination hotspots, the role of weeds and crops as reservoirs of viruses, the role of informal germplasm exchange in the spread of viruses and/or evidence for plant-to-plant transmission of BanMMV in La Réunion.

6. Conclusions and recommendations

In conclusion, our study represents a significant advancement and provides crucial information on the prevalence and diversity of viruses infecting banana plants in La Réunion. Furthermore, our results show that banana viruses have a very low prevalence and do not seem to be a major issue for banana production in La Reunion. However, it has certain limitations due to its small sample size and lack of diversity. In addition, the methodological improvements carried out during our study appear promising, but further studies, testing and validation could be carried out to validate our results and to determine the performance characteristics and ensure the reliability of the diagnostic improvements made during our study. For this purpose, methodological comparisons and enhancements should be performed and replicated on a larger sample panel than the one we studied. Besides, the validation of these methods could also be the subject of inter-laboratory comparison studies.

Based on the results of our study, it would be interesting for future investigations to conduct in-depth sampling in the areas of origin of the diseased plants to determine whether they are isolated cases or contamination hotspots. Additionally, if other positive cases are detected, it would allow for a more extensive diversity study and a better understanding or confirmation of virus transmission mechanisms, such as plant-to-plant transmission.

Moreover, larger-scale sampling should cover the entire island and include a greater diversity of production plots, especially those in polyculture, to determine the transmission between CMV hosts and banana plants and the prevalence of this virus in bananas.

Furthermore, testing virus presence on banana plants from private gardens would be valuable, as they could also play a significant role as reservoir of virus transmission. Implementing participatory sampling could facilitate this approach.

7. Annexes

	A	B	C	D	E	F	G	H	I	G	K
1	■			■			■			■	
2		■			■			■			■
3			■			■			■		
4	■			■			■			■	
5		■			■			■			■
6			■			■			■		
7	■			■			■			■	
8		■			■			■			■
9			■			■			■		
10	■			■			■			■	
11		■			■			■			■
12			■			■			■		
13	■			■			■			■	
14		■			■			■			■

Supplementary Figure 1: Template used for stratified sampling in banana production plots.

Within a square of 11 x 14 plants positioned randomly within the plot, one every three plant was sampled in each row. Green boxes correspond to sampled plants.

450	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
451	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
452	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
453	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
454	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
455	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
456	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
457	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
458	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
459	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
460	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
461	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
462	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	380	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA
463	Grande Naine	05-09-2023	Saint-Philippe	Rue Aime Turpi	Cultivée	-21,354474	55,634791	291	Rejet	NA	2	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	NEGATIF	AAA

Supplementary Table 1 : Summary table of sampling data and indexing results for each sample collected during this prevalence study.

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